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# BIOLOGICAL EFFECTS OF HEAVY WATER

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The observations of Lewis (1933) on the germination of seeds and of Taylor, Swingle, Eyring, and Frost (1933) on various fresh water animals have indicated that concentrated deuterium water or heavy water is toxic for, or at least unfavorably affects living matter. The cause of this action is at present unknown, but certain possibilities can be eliminated by the experiments which are reported in this paper. It is obvious that a mass of information must be gathered before interpretation of results becomes possible, and the following observations are frankly exploratory.

The water used was 95 to 97 per cent heavy water, kindly supplied by Professor H. S. Taylor of the Chemistry Department.

Because of the extreme sensitivity of living organisms to small amounts of impurities of various kinds, great attention was paid to the purification and testing of the samples of heavy water used. In the various stages of preparation of heavy water from electrolysis and burning of the hydrogen and oxygen formed, there is possibility of oxidized hydrocarbons, possibly aldehydes (from ozone acting on rubber tubing), being formed and also readily detectable amounts of nitrous acid from the oxyhydrogen flame burning in air. Water so formed is toxic to *Paramœcia,* killing them instantly with discharge of trichocysts, whether it contains 97 per cent heavy water or only 0.2 per cent heavy water, an effect undoubtedly due to its acidity. If redistilled from alkali alone or from alkali and $KMnO_4$, the 0.2 per cent heavy water is harmless while the 97 per cent heavy water kills *Paramœcium* in 6 to 15 hours, without discharge of trichocysts. The 0.2 per cent heavy water formed by oxyhydrogen combustion and redistilled from alkali and permanganate is also non-toxic to *Euglena, Amœba, Epistylis,* luminous bacteria and other small organisms, so that it appears reasonably certain that any effects of concentrated heavy water treated in the

same way must be due to the deuterium it contains and not to unknown impurities. There is always a possibility of doubt that minute traces of some unknown impurity may be present in a minimal effective concentration, but all known precautions have been taken to remove such. In the experiments reported below, heavy water redistilled from alkali has always been used and in most cases heavy water redistilled from alkali and permanganate.

### Effect on Luminescence

The dry powdered luminous organism, *Cypridina*, when added to 97 per cent heavy water luminesces just as brightly and with the same color as in distilled water.

A suspension of luminous bacteria[1] (*Vibrio phosphorescens*, a fresh water form) luminesces about as brightly in approximately 85 per cent heavy water as in distilled water and the light lasts for over 24 hours in each, becoming less and less bright. After five days the bacteria in both heavy and distilled water, when inoculated in culture media in ordinary water, grew and luminesced normally. When inoculated in culture media in ordinary water after 41 days in heavy water, the bacteria also grew and luminesced normally. It is quite evident that the heavy water does not kill these forms.

Experiments with a salt water bacterium, isolated from squid at Woods Hole, Mass., added to heavy water plus salt (about 85 per cent final concentration), showed a marked diminution of luminescence in the 85 per cent heavy water after 4 hours as compared with ordinary water. In another experiment 63 per cent heavy water dimmed in 3 hours, but 36 per cent heavy water did not appreciably. The luminescence of the salt water bacterium is undoubtedly affected in the heavy water.

### Effect on Growth of Luminous Bacteria

The next experiment was devised to find out if luminous bacteria would grow in a culture medium made up in 97 per cent heavy water. Two kinds of bacteria were also used, the fresh water form (*Vibrio phosphorescens*) and the salt water form. The sterile culture medium consisted of: 23 mg. bactonutrient agar, 10 mg. glycerine, 1 mg. $CaCO_3$ per cc. For the salt water form 30 mg. table salt was added, and for the fresh water form 3 mg. table salt. In one experiment with the fresh water form there was some growth and luminescence, while in two other experiments there was some growth but none or very faint luminescence.[2]

[1] I am deeply indebted to Mr. I. M. Korr for cultures of both forms of luminous bacteria.

[2] Frequently luminous bacteria will grow but will not luminesce under adverse conditions such as treatment with ultra-violet light or high temperatures (38°).

There was good growth and luminescence on the ordinary water controls.

Using the salt water form, in three experiments carried out at different times, a good diffuse [3] luminescent growth occurred in the 94–97 per cent heavy water medium, whereas a bright streak of growth and luminescence appeared on the ordinary water medium. After 24 days bacteria were transplanted from the heavy and ordinary water media to new ordinary water culture media and they grew and luminesced.

The conclusions are that luminous bacteria will grow slowly and one species will luminesce in 97 per cent heavy water, but not as well as in ordinary water, and that they are not killed by long contact with heavy water.

### Effect on Protozoa and Small Organisms

Although *Paramœcium* and *Euglena* were studied by Taylor, Swingle, Eyring and Frost (1933), new samples of 95 per cent, 97 per cent and 100 per cent heavy water were tested on these forms and others to see if various samples of heavy water would give the same effects. It was found that they did. The experiments were carried out in hanging drops on cover glasses on depression slides sealed with vaseline. The drop of heavy water was mixed with a minute drop of water containing the organisms. This dilutes the 97 per cent heavy water to an unknown amount, possibly to 85–90 per cent.

*Paramœcia* almost immediately give the avoiding reaction and swim slowly in the heavy water, soon appearing somewhat bloated, and in two hours the contractile vacuoles are enormously enlarged and fail to empty. The well-known blisters appear at the surface. The animals are dead and disintegrated in less than 24 hours, while controls in distilled water are normal after 5 days.

One experiment was run with 100 per cent heavy water (somewhat diluted by the *Paramœcia*). They were killed in 6 to 10 hours with a similar sequence of events, while they live for days in 0.2 per cent heavy water. *Paramœcia* never recover in ordinary water when once disintegrated.

*Amœba dubia* and two rotifers, *Monostyla bulba,* and *Philodina roseola,* in heavy water are killed in from 6 to 20 hours. The *Amœbæ* round up, show no movement (and no large contractile vacuole) and look disintegrated in about six hours, whereas in distilled water, the *Amœbæ,* although rounded up at first, are quite normal after 48 hours, as are also the rotifers. The same statement applies also to another large species of *Amœba* and to a *Vorticella*-like infusorian, *Epistylis,* which almost immediately withdraws its peristome, the cilia beat more

[3] Although a streak inoculation was made.

and more slowly and soon all movement ceases and disintegration occurs. Controls are normal after 48 hours.

The conclusion is that a large number of small organisms are killed in 85–90 per cent heavy water and the more rapidly the greater its concentration.

### Recovery of Euglenæ

*Euglena gracilis* in 97 per cent (slightly diluted) heavy water mostly rounds up and remains immobile for days. On the ninth day, the heavy water was replaced by distilled water and most of the *Euglenæ* recovered and swam about perfectly normally.

*Euglena proxima*[4] also shows the avoiding reaction, (euglenoid shapes), mostly round up and become immobile in heavy (90 per cent) water, but a certain percentage may be motile for three days although not nearly as active nor as elongate as the controls in ordinary water or water containing 0.2 per cent $D_2O$. After three days movement in practically all the organisms has ceased. After five days ordinary water was added to greatly dilute the heavy water and most of the *Euglenæ* recovered completely.

The conclusion is that *Euglenæ* are not irreversibly injured by the heavy water.

### Effect on Protoplasmic Rotation

One-half of an *Elodea* leaf, showing marked protoplasmic rotation, was placed in 95 per cent heavy water at 25° C. A slowing of rotation occurs at first which very soon begins again, and in 15 minutes rotation is nearly as marked as in the half control leaf in ordinary distilled water at 25°. The heavy water was then replaced by more 95 per cent heavy water and this was again replaced after two hours so that the leaf must finally have been exposed to approximately 95 per cent heavy water with no dilution. The rotation gradually slows over a period of hours and there is still some slow rotation in some cells after 5 hours but much less than in the control. After 24 hours there was still some slow rotation in the leaf in heavy water but practically none in the control in distilled water, but rotation began again later. Another experiment gave a similar result. The cells look quite normal in appearance.

The conclusion is that a gradual slowing of protoplasmic rotation occurs in concentrated heavy water but that it is not marked or rapid and that *Elodea* cells may show rotation and are not injured by heavy water after 24 hours.

[4] Kindly supplied in pure culture by Dr. R. Glaser of the Rockefeller Institute, Princeton, New Jersey.

### Penetration of Heavy Water into Cells

In order to test penetration in a rough way, *Elodea* leaf cells were plasmolyzed in m/2 cane sugar solution and then returned to heavy water, when the protoplasts reëxpand again. The heavy water must have entered the *Elodea* cells to again restore osmotic equilibrium. This method is not sufficient to detect differences in rate of entrance as compared with ordinary water, which should be carried out on the swelling of spherical cells such as *Arbacia* eggs in a hypotonic medium. Effects of heavy water cannot be attributed to non-penetration. Indeed the enormous enlargement of the contractile vacuoles in *Paramœcium* in heavy water points to an interference with the water-eliminating mechanism rather than an influence on water penetration. However, such an interference may be brought about by many unfavorable conditions.

### Does $H_2O_2$ Accumulate in Heavy Water?

In view of observations of Taylor and Pace[5] that heavy water retards the action of liver catalase on $H_2O_2$, experiments were carried out to determine if the effect of heavy water is so to retard the action of catalase in cells as to cause possible accumulation of $H_2O_2$ as a result of respiration or possibly of photosynthesis. In either case the destructive effect might be due to accumulation of $H_2O_2$.

This theory can be tested in three ways. First, by finding if heavy water is toxic for anærobic forms that contain no catalase. Second, by testing ærobic forms, which can also live under anerobic conditions for some time, in absence of oxygen. Third, by testing green organisms like *Euglena* in light and in darkness.

*Euglena proxima* or *E. gracilis* was placed in 85–90 per cent heavy water on slides in hanging drops and kept both in darkness and in light. Since this concentration of heavy water does not immediately affect all the organisms but merely causes many of them to become spherical and immobile, while others move about slowly, any additional $H_2O_2$, possibly accumulating in light from photosynthetic processes, might be sufficient to cause a difference in behavior of the organism in light and in darkness. However, no significant difference could be found, although many experiments were performed. In heavy water spherical and immobile *Euglenæ* do not become active in the dark. After three days all the *Euglenæ* were immobile and spherical whether they had been kept in light or in darkness. In general, *Euglenæ* are more active in the dark, distributing themselves uniformly through the drop, whereas in the light they collect in one spot, but this is a light reaction. It occurs in ordinary

[5] Private communication from Professor H. S. Taylor.

water also and cannot be connected with the possible accumulation of $H_2O_2$.

*Paramœcium* cannot be tested in heavy water in a pure hydrogen atmosphere because *Paramœcium* in culture medium will not withstand absence of oxygen for any length of time. They are mostly killed in one hour in a hanging culture drop in a pure hydrogen atmosphere.

However, if *Paramœcia* are killed by the accumulation of $H_2O_2$ in heavy water, the effects of $H_2O_2$ on *Paramœcia* should be the same as the effects of heavy water. To test this, Merck's superoxol (a very pure preparation of $H_2O_2$) was added to culture medium containing *Paramœcium* in a concentration which caused disruption and surface blister formation in 1 to 1.5 hours. Only a slight enlargement of the contractile vacuole occurred, but not the enormous enlargement characteristic of a heavy water effect. This may be taken as additional evidence against the view that accumulating $H_2O_2$ is responsible for the toxic effects on *Paramœcium*.

## Summary

Heavy water (85–95 per cent) has been found not to prevent the luminescence of dried *Cypridina* nor to affect the luminescence of a fresh water luminous bacterium but to diminish the luminescence of a marine form; to retard growth of luminous bacteria, sometimes allowing slow growth without luminescence; to kill a number of protozoa and rotifers, but not to kill bacteria and not to injure *Euglena* irreversibly; to affect *Euglena* equally in light and in the dark; to affect only slowly protoplasmic rotation of *Elodea* cells and to penetrate into *Elodea* cells. In view of the slow and often reversible effects of heavy water, its action may be likened to that of a generally unfavorable environment, leading to progressive changes in the cell. No more can be said at present than to suggest that these changes are the result of differential effects on the rate of biochemical reactions which ordinarily proceed at a certain definite rate in relation to each other.

## LITERATURE CITED

Lewis, G. N., 1933. The Biochemistry of Water containing Hydrogen Isotope. *Jour. Am. Chem. Soc.*, **55**: 3503.

Lewis, G. N., 1934. The Biology of Heavy Water. *Science*, **79**: 151.

Taylor, H. S., W. W. Swingle, H. Eyring, and A. A. Frost, 1933. The Effect of Water Containing the Isotope of Hydrogen upon Fresh Water Organisms. *Jour. Chem. Phys.*, **1**: 751.

# THE EFFECTS OF LAND DRAINAGE UPON THE EXCESS BASES OF SEA WATER[1]

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The total titratable alkalinity of sea water, that is, its buffer capacity for acids, tends to reach an equilibrium such that the total buffer capacity divided by the chlorinity is a constant. This fact has been established by several investigators working on samples from the North Sea and its vicinity and by Thompson and Bonnar (1931), who studied the waters of the region of the San Juan archipelago and Puget Sound. The results of Mitchell and Rakestraw (1933) on Atlantic waters of the Cape Cod and Bermuda regions also revealed the tendency toward constancy of ratio. It is to be expected that when the only important change in the sea water composition is that due to the addition or withdrawal of water by rain or evaporation, this and similar ratios should be constant.

It appears, however, that the ratio as observed in different regions is not necessarily the same. The differences might be due, in part at least, to variations in methods of measurement but this appears not to be the entire explanation (Mitchell and Rakestraw, 1933). It would seem, then, that the oceans are not in apparent equilibrium as regards the ratio of excess base to the salts. But although some approach to a constant buffer capacity-chlorinity ratio is discernible in ocean waters, such is not the case, generally speaking, in the waters of sounds, bays and inlets where equilibrium conditions are more actively disturbed.

Thompson and Bonnar have discussed factors which alter the ratio. Among them are photosynthesis and other effects of plant life and of animal life and the effect of land drainage. Our own experience has shown the latter to be a very significant factor tending, as Thompson and Bonnar found, to raise the buffer capacity-chlorinity ratio but showing, in our observations, great variability with locality, character of the soil and rapidity of drainage, that is, whether surface or deep drainage. It has even appeared possible to us that the character of the soil in different regions of the earth might vary sufficiently in potentiality for yielding bases to the sea to account in part for the differences in the ratio as apparently found in different ocean areas.

[1] Contribution No. 42 from the Woods Hole Oceanographic Institution.

A satisfactory illustration of the land drainage effect was obtained by observations on Narragansett Bay. It is a relatively long and narrow body of salt water. No large, long rivers enter it so that the drainage which it receives is of comparatively local origin. Measurements of buffer capacity were made by a method described in a previous communication (Mitchell and Rakestraw, 1933). The results of typical observations, as shown in Table I, reveal what is probably demonstrable for any similar body of water, namely, that the lower the salinity the higher, in general, is the buffer capacity-chlorinity ratio. This is a tendency rather than an exact relationship because of the disturbing

TABLE I

*The Relative Buffer Capacity of Narragansett Bay Water Samples Arranged in Decreasing Order of Chlorinity*

| Chlorinity *grams per liter* | Ratio $\frac{BC}{Cl}$ | Chlorinity *grams per liter* | Ratio $\frac{BC}{Cl}$ |
|---|---|---|---|
| 19.50 | 0.1143 | 15.94 | 0.1272 |
| 19.00 | 0.1153 | 15.93 | 0.1242 |
| 18.10 | 0.1222 | 15.50 | 0.1191 |
| 17.90 | 0.1227 | 15.50 | 0.1216 |
| 17.38 | 0.1210 | 15.40 | 0.1275 |
| 17.38 | 0.1217 | 15.22 | 0.1266 |
| 17.02 | 0.1208 | 15.05 | 0.1307 |
| 17.02 | 0.1208 | 14.96 | 0.1229 |
| 16.85 | 0.1189 | 14.60 | 0.1283 |
| 16.84 | 0.1171 | 14.52 | 0.1383 |
| 16.73 | 0.1186 | 14.45 | 0.1338 |
| 16.72 | 0.1239 | 14.06 | 0.1345 |
| 16.55 | 0.1200 | 13.88 | 0.1364 |
| 16.48 | 0.1223 | 13.15 | 0.1258 |
| 16.36 | 0.1214 | 13.04 | 0.1425 |
| 16.31 | 0.1251 | 11.92 | 0.1535 |
| 16.20 | 0.1258 | 5.40 | 0.1630 |
| 16.16 | 0.1237 | 3.61 | 0.1690 |
| 16.05 | 0.1188 | | |

effects of tidal currents, storms, freshets and local conditions such as the relative abundance of plant and animal life.

In order to demonstrate more clearly the relationship between dilution of estuary waters and their buffer capacities, observations were made upon various series of mixtures of salt water with fresh water from selected sources. In every case progressive dilution caused a rise in the buffer capacity-chlorinity ratio such that the latter plotted as ordinates against the chlorinity as abscissas gave a smooth curve. The rise in the ratio with addition of fresh water was not, however, the same in all cases. To illustrate typical variations, a number of the curves obtained are shown in Figs. 1 and 2.

Curves *A*, *B* and *C* were obtained by dilution of Narragansett Bay water with that of the Pawtuxet River which drains the watershed supplying the city of Providence. The fact that observations on the undiluted salt water from the bay, giving the lowest point of each curve, do not yield closely approximating results is due to the circumstance that water collections were made at different stages of the tide and in one case (*A*) shortly after an easterly storm which raised the salinity of the water above its usual value. This is in part the explanation of the different levels of the curves. But it may also be seen that the slopes of the curves are different.

Curve *A*, obtained on March 7, 1933, illustrates the effect of an easterly storm in that the undiluted bay water has a relatively high

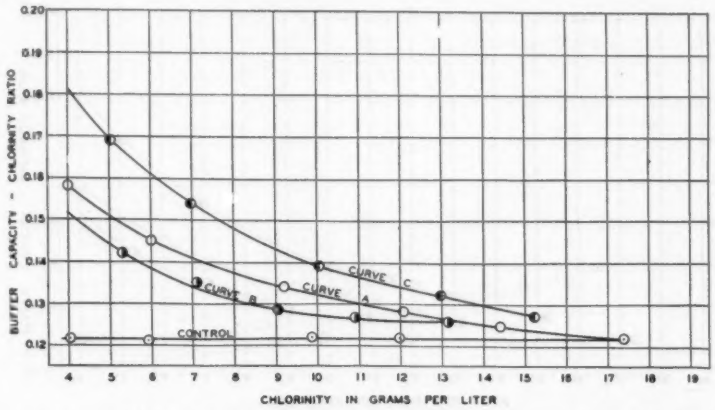


FIG. 1. Effect of successive dilutions of Narragansett Bay water with Pawtuxet River water. Curve *A*, in March, Curve *B*, after a freshet in April, Curve *C*, in June. For the control, dilutions were made with distilled water.

salinity and a low ratio. The rise in the ratio with progressive dilution is an intermediate one increasing from 0.1253 (Cl, 14) to 0.1583 (Cl, 4).

Curve *B*, obtained on April 11, 1933, illustrates the effect of mere surface drainage. There had been a light snowfall followed by especially heavy and prolonged rain. The river was at spring freshet level. The ratio increased only from 0.1252 (Cl, 14) to 0.1520 (Cl, 4).

Curve *C*, however, illustrates the more typical effect of the true soil drainage of this area. Sampled on June 13, 1933, after a period of approximately normal rainfall, the river water raised the ratio from 0.1293 (Cl, 14) to 0.1820 (Cl, 4).

There is then a seasonal variation in land drainage effects upon the

titratable base of estuary waters. The larger the proportion of deep soil drainage to surface run-off, the greater is the effect in this particular type of drainage area.

Curves *D* and *E* illustrate the relatively large effect of deep soil drainage in another way. The salt water in these cases came from Sakonnet Point at the more easterly of the two mouths of Narragansett Bay. The fresh water in the case of curve *D* was taken from a pond about one mile inland from the Sakonnet shore and in the case of curve *E* from a clear deep spring near the edge of the same pond. The ratio in curve *D* increased from 0.1240 (Cl, 16) to 0.1381 (Cl, 6) while in curve *E* the increase was much greater, from 0.1182 (Cl, 16) to 0.1490 (Cl, 6).

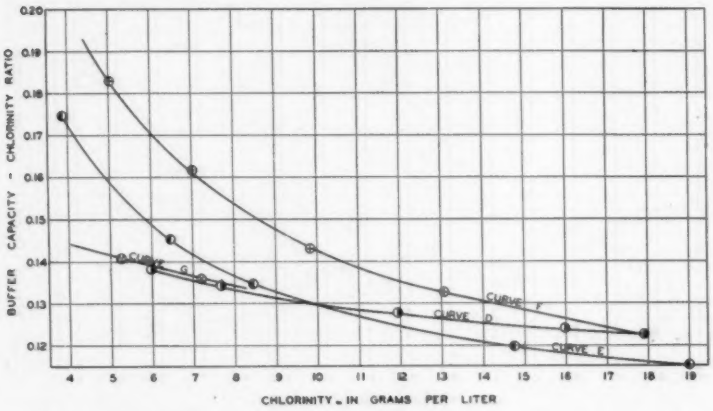


FIG. 2. Effect of successive dilutions of sea water with fresh water from various sources. Curve *D*, with pond water, Curve *E*, with spring water, Curve *F*, with pond water from swampy soil, Curve *G*, with pond water from sandy soil.

Another factor influencing the drainage effect is the character of the soil. Curves *F* and *G* illustrate this fact. The sea water used for both of these sets of measurements was taken from Woods Hole Harbor on June 14, 1933, after a period of only moderate rainfall. The fresh water used for the dilutions of curve *F* was collected on the same day from a nearby pond in a swampy area, while that used for curve *G* came from another pond about four miles away entirely surrounded by very sandy soil. Curve *F* shows a rise of the ratio to 0.1832 (Cl, 5) but curve *G* rises only to a ratio of 0.1416 at the same dilution. Apparently a barren, sandy soil yields comparatively little excess base to its drainage water.

The curve at the bottom of Fig. 1 indicates data for a control upon the accuracy of the measurements and the reproducibility of the results. It was obtained by adding distilled water to a sample of Narragansett Bay water. It shows no change, in excess of the limits of observational error, in the ratio even when the chlorinity is diluted to 4 grams per liter. The probable error of a single determination of the ratio is found to be approximately $\pm$ 0.0007, a value in accord with numerous other estimations of the probable error of this method.

## Summary

1. Land drainage tends, in general, to raise the buffer capacity-chlorinity ratio of sea water.

2. Heavy rains measurably decrease the proportional effect.

3. The effects of deep soil drainage are greater in raising the ratio than are those of mere surface drainage.

4. The character of the soil alters the effect. Sandy soil may yield measurably less base than a richer soil.

## REFERENCES

Mitchell, P. H., and N. W. Rakestraw, 1933. The Buffer Capacity of Sea Water. *Biol. Bull.*, **65**: 437.

Thompson, T. G., and R. U. Bonnar, 1931. The Buffer Capacity of Sea Water. *Ind. Eng. Chem.*, Anal. Ed., **3**: 393.

# PARADOX PHENOMENA IN THE CARDIAC GANGLION OF LIMULUS POLYPHEMUS[1]

IPING CHAO[2]

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It was found by Zwaardemaker (1917, 1925) that when a frog's heart is perfused with a Ringer's solution containing 40 mgm. thorium nitrate per liter instead of the 170 mgm. potassium chloride normally present, a temporary arrest of the beat occurs at the moment of replacing the thorium-containing by the potassium-containing Ringer's solution. This temporary inhibition of the activity of the heart on replacing a light radioactive element (potassium and rubidium in one group) by a heavy radioactive element (uranium, thorium, radium, emanation and ionium in another group), or vice versa, was called by Zwaardemaker the radio-physiologic paradox. Shortly afterwards Libbrecht (1920) found that the same temporary inhibition of the frog's heart can be obtained on change from a K-free to a normal Ringer's solution, and he called this striking phenomenon the potassium-paradox. Libbrecht (1921) also described a thermo-paradox in the frog's heart on change from a warm to a cold Ringer's solution. More recently Kisch (1930) reported a Ca-paradox on change from a Ca-free to a normal Ringer's solution and a Sr-paradox on change from a Ca-free to a normal Ringer's solution containing an equivalent amount of $SrCl_2$ instead of $CaCl_2$.

Paradoxes of this kind are found not only in the frog's heart but also in the hearts of other animals and in other tissues. Thus the K-paradox has been described in the rabbit's heart by Busquet (1921, 1922*b*) and in the heart of the spider crab by Wells (1928). Busquet (1922*a*) also observed a similar paradox in the rabbit's heart on change from a K-free Ringer's solution to the same solution containing ammonium chloride. The K-paradox has been demonstrated in the automatic movement of the frog's œsophagus by Bakker (1927) and in the isolated fore-gut of insect by Hobson (1928). It is also found in the ganglion of the *Limulus* heart (Chao, 1933). In this paper a further study of the conditions

[1] A preliminary report of this work appeared in the *Collecting Net*, 1933, **8**: 341.

[2] Partly aided by a Collecting Net Scholarship.

for the production of the K-paradox in this ganglion will be presented, together with observations on the Ca-paradox and thermo-paradox.

The experimental procedure has been described in a previous paper (Chao, 1933). Briefly, the ganglion is isolated from the heart musculature posteriorly and the heart is sectioned behind the second segment, leaving the ganglion in connection with the anterior two segments of the heart only. The heart is mounted in sea water for graphic registration of the contractions, which result from impulses originating in the nerve cells of the ganglion; the latter is immersed in a Ringer's solution. The Ringer's solution contains 445 millimols NaCl, 8.9 millimols KCl and 37 millimols $CaCl_2$ in a liter. It can be rapidly replaced by another solution without disturbing the ganglion-heart preparation. All experiments (except those on the thermo-paradox) are performed at the room temperature; this fluctuates usually within one degree during an experimental period of two to three hours, and varies between 22° to 26° C. during the whole season.

## The Potassium-Paradox

When a ganglion is immersed in a K-free Ringer's solution, the rate is slightly increased. Ten minutes later the K-free Ringer's solution is replaced by a normal Ringer's solution;[3] the rate and amplitude, instead of returning gradually to normal, are immediately decreased, and in some cases the heart-beat may be even completely arrested. This temporary inhibition is the characteristic phenomenon of the paradox; it lasts for a short period and is followed by a gradual recovery. The extent of the inhibition depends upon several factors, which will be discussed in order.

### *The Effect of Repeated Immersion in the K-free Ringer's Solution*

In general, the K-paradox is more readily obtained on a second or third repetition than on the first immersion of a fresh ganglion in the K-free Ringer's solution. The following experiment (No. 86), although an extreme case, illustrates this point particularly well. When this ganglion was first immersed in a K-free Ringer's solution for ten minutes, no K-paradox was obtained on return to the normal Ringer's solution. Twenty minutes later the ganglion was again immersed in a K-free Ringer's solution for another ten minutes. Then on change of solutions the rate decreased from 15.5 beats per minute (the rate at the tenth minute in the K-free Ringer's solution) to 5, 6, 6, 8, 8, 8, 8, 9, 9,

[3] The K-paradox can also be demonstrated on changing from a K-free Ringer's solution to sea water.

and 9 in successive minutes in the normal Ringer's solution; after twenty minutes the rate was 9.5. Corresponding to the primary decrease in rate, there was also a slight decrease in amplitude (Fig. 1, *a*). These changes in rate and amplitude were even more pronounced after the third immersion in the K-free Ringer's solution for the same length of

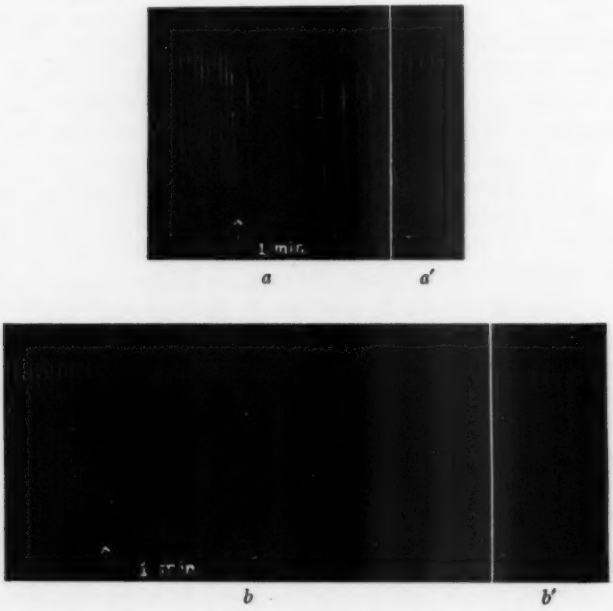


FIG. 1. Experiment No. 86. Effect of repeated immersion in the K-free Ringer's solution on the production of the K-paradox. (*a*) After a second immersion in a K-free Ringer's solution for ten minutes the ganglion, which did not give any sign of the K-paradox on the first trial, was returned to a normal Ringer's solution at the point marked by an arrow. (*a'*) Sixteen minutes later in the normal Ringer's solution. (*b*) K-paradox obtained on a similar immersion for the third time. (*b'*) Twelve minutes later in the normal Ringer's solution. All figures read from left to right, and the change of solution is always marked by an arrow.

time. On transfer to the normal Ringer's solution the heart beat was immediately arrested for a minute and a half (Fig. 1, *b*).

In order to evaluate quantitatively the paradoxical effects of these two trials, the average rate for the first five minutes immediately following the change from the K-free to the normal Ringer's solution ($R'$) was compared with the final rate in the latter ($R$) when recovery is

complete. Expressed as the average percentage decrease in rate for the first five minutes following the change of solutions $[(R - R')/R \times 100]$, the results of these trials are given in Table I.

TABLE I

*Average percentage decrease in rate for the first five minutes on return to the normal Ringer's solution after repeated immersions in the K-free Ringer's solution.* (Experiment No. 86.)

| Number of trials | Percentage decrease in rate |
|---|---|
| First | 0 |
| Second | 31 |
| Third | 74 |

In this manner the intensity of the K-paradox is conveniently expressed in any experiment in terms of the primary decrease in rate. The greater the proportional decrease in rate, the more pronounced is the paradoxical effect.

Since the paradoxical effect is increased with the repeated immersion in the K-free Ringer's solution, a difficulty arises in comparing instances of K-paradox obtained under different conditions, even when the same ganglion is used. The difficulty may be obviated by performing the experiments in pairs, the order of the successive trials being reversed in the two or three trials of each pair.

TABLE II

*Average percentage decrease in rate for the first five minutes on return of the ganglion to normal Ringer's solution after immersions of unequal durations in K-free Ringer's solution.*

| Duration of immersion in the K-free solution | Decrease in rate | | | Average |
|---|---|---|---|---|
| | Exper. No. 9 | Exper. No. 10 | Exper. No. 11 | |
| *minutes* | *per cent* | *per cent* | *per cent* | |
| 10 | 7.5 | 20 | 28 | 19 |
| 20 | 23 | — | 31 | 27 |
| 30 | 35 | 39 | — | 37 |

### *The Effect of the Duration of Immersion in the K-free Ringer's Solution*

A certain minimum duration of immersion in the K-free Ringer's solution is necessary for the production of the K-paradox. This minimum duration, however, is difficult to determine with exactitude for the above-mentioned reason. On the other hand, if the exposure to the

K-free Ringer's solution is too long, so as to cause irreversible change in the ganglion, the rate and amplitude may continue to decrease on return to the normal Ringer's solution and no recovery will be seen. Within certain limits, up to an immersion of about thirty minutes, the changes are perfectly reversible in a good ganglion. Table II gives the comparison of the K-paradox obtained after immersions of 10, 20, and 30 minutes in the K-free Ringer's solution for three different ganglia.

In experiment No. 9 the order of experimentation was from shorter to longer durations; in experiments Nos. 10 and 11, from longer to shorter durations. As the duration of immersion in the K-free Ringer's solution increases, the decrease in rate becomes greater, and the primary decrease in amplitude is more pronounced; also the time for complete recovery is more prolonged.

*The Effect of the Difference in K-content in the Two Solutions*

Between the K-free and the normal Ringer's solution the difference of the K-content is about 9 millimols per liter, and the K-paradox usually appears on transfer to the normal Ringer's solution after an immersion of 10 minutes in the K-free solution. (In most of the experiments an immersion of ten minutes was adopted as the standard duration.) The paradox is also seen when a ganglion is transferred from the K-free solution to one containing an excess of KCl (*e.g.*, 18 millimols per liter). Although the excess of KCl by itself produces a rapid rhythm, yet the primary inhibition, particularly of the amplitude, is still obvious. In general, the paradoxical effect obtained on change from the K-free Ringer's solution increases, within certain limits, with the K-content of the second solution.[4]

When a K-poor Ringer's solution is used (*e.g.*, one containing 1 or 2 millimols KCl per liter) the K-paradox is still obtained on change to a normal Ringer's solution, or to one containing an excess of KCl. It is also obtained on change from a normal Ringer's solution to a Ringer's solution containing a large excess of KCl, about 70 to 100 millimols per liter. In general, a relatively great difference in the K-content of the two solutions is necessary for the production of the K-paradox, when the change is from a normal Ringer's solution to one containing an excess of KCl. The paradoxical effect is thus not directly proportional to the difference in the K-content. It is important to note that no such paradoxical effect is ever observed when the K-content is changed in the *decreasing* direction (*e.g.*, change from a K-excess to a normal Ringer's

[4] The K-paradox can also be observed on change to a Ringer's solution containing only 1 millimol KCl per liter after an immersion in the K-free Ringer's solution for twenty minutes.

solution, or from a normal to a K-poor Ringer's solution). The determining factor for the K-paradox is always the *sudden increase* in the K-content.

*The Effect of the Presence of Other Electrolytes*

When a fresh ganglion is immersed in a pure isotonic sucrose solution the rhythm can be maintained quite well for a considerable period up to half an hour; the precise duration depending upon the condition of the ganglion. If the ganglion thus active in pure sucrose solution is transferred to a sucrose solution containing KCl in the concentration normally present in the Ringer's solution, it is found that both the rate and the amplitude of the beat undergo a continuous decrease and the rhythm is gradually arrested in a few minutes; no K-paradox can be seen. This observation indicates that the presence of electrolytes other than KCl, either in the K-free or in the K-containing solution or in both, is necessary for the production of the K-paradox. There are thus two electrolytes ($CaCl_2$ and KCl) and two solutions (the K-free and the K-containing solutions) to be considered.

*Is $CaCl_2$ Necessary for the Production of the K-paradox?*—The K-paradox can be readily obtained on change from a pure isotonic NaCl solution to a normal Ringer's solution. Calcium is thus not necessary in the K-free solution. It is also not necessary in the K-containing solution, for the K-paradox is obtained on transfer from a K-free to a Ca-free Ringer's solution. The K-paradox appears on change from a pure isotonic NaCl solution to a Ca-free Ringer's solution (Fig. 2).

FIG. 2. Experiment No. 79. K-paradox obtained on change from a pure isotonic NaCl solution to the same containing 9 millimols KCl per liter (*i.e.*, the Ca-free Ringer's solution).

This last observation shows conclusively that $CaCl_2$ is not necessary for the production of the K-paradox and can be omitted from both the K-free and the K-containing solutions.

It should be noted, however, that in the last experiment cited, where the K-paradox appears on change from a pure NaCl solution to a Ca-

free Ringer's solution, the rate is only slightly inhibited for a short period and the primary decrease in amplitude may not appear at all. The difficulty with which the K-paradox is obtained under these circumstances is due to the fact that both the pure NaCl and the Ca-free solutions normally give rise to a rapid and irregular rhythm.

On the other hand, when an excess of $CaCl_2$ (*e.g.*, 100 millimols per liter) is present in the K-free solution, the K-paradox can still be obtained on return to the normal Ringer's solution if the rate and amplitude are not unduly decreased while the ganglion is in the former solution. Similarly, the K-paradox still can be obtained on change from a K-free Ringer's solution to a normal Ringer's solution containing an excess of $CaCl_2$ (100 millimols per liter), provided the $CaCl_2$ does not reach such a concentration as to inhibit the rhythm by itself (Fig. 3). Finally the K-paradox has been obtained on change from a K-free to a

FIG. 3. Experiment No. 92. K-paradox obtained on change from a K-free Ringer's solution to a normal Ringer's solution containing an excess of $CaCl_2$ (100 millimols per liter).

K-containing Ringer's solution, both containing the same excess of $CaCl_2$ (80 millimols per liter). It seems, therefore, that the K-paradox is not abolished by the removal of $CaCl_2$, nor is it antagonized by the excess of $CaCl_2$, and that the general effect of $CaCl_2$ is only secondary, consisting in balancing part of the toxic action of NaCl.

*Is NaCl Necessary for the Production of the K-paradox?*—Since the presence of some other electrolyte either in the K-free or in the K-containing solution has been shown to be necessary for the production of the K-paradox, and since $CaCl_2$ has been shown to be unnecessary, the logical conclusion follows that NaCl must be the necessary electrolyte. In fact, the paradoxical effect is found to be progressively diminished with progressive decrease in the NaCl-content of the K-free solution. When a fresh ganglion is immersed in a mixture of 30 volumes of a K-free Ringer's solution [5] and 70 volumes of isotonic sucrose

[5] K-free Ringer's solution is used instead of pure NaCl solution on account of the less toxic nature of the former.

solution, hardly any sign of the K-paradox can be demonstrated on return to the normal Ringer's solution. The K-paradox is obtained only on a second or third trial with such a solution, and the paradoxical effect is always less marked than that obtained with a 100 per cent K-free Ringer's solution. Table III gives the results of the primary decrease in rate for four experiments. In these experiments the first trials with either the 100 per cent or the 30 per cent K-free Ringer's solution were discarded, and in two experiments the order of treatment with the two solutions was reversed for the purpose of control, as already described.

When NaCl is entirely replaced by sucrose in the K-free solution the K-paradox is entirely abolished. No paradoxical effect has ever been

TABLE III

*Average percentage decrease in rate for the first five minutes on change to normal Ringer's solution after an immersion of ten minutes in (1) a 100 per cent and (2) a 30 percent K-free Ringer's solution.*

| Experiment No. | Decrease in 100 per cent solution *per cent* | Decrease in 30 per cent solution *per cent* |
|---|---|---|
| 75 | 36 | 29 |
| 76 | 36 | 20 |
| 77 | 33 | 18 |
| 78 | 54 | 16 |
| Average | 40 | 21 |

observed on change from a pure isotonic sucrose solution, or from the same solution containing 37 millimols $CaCl_2$ in a liter, to the normal Ringer's solution.

NaCl, however, is necessary only in the K-free Ringer's solution, for the K-paradox can be obtained on change from a pure isotonic NaCl

FIG. 4. Experiment No. 63. K-paradox obtained on change from a pure isotonic NaCl solution to a sucrose solution containing 9 millimols KCl per liter.

solution to a sucrose solution containing KCl in the concentration normally present in Ringer's solution [6] (Fig. 4).

*The Calcium-paradox*

When a ganglion is immersed in a Ca-free Ringer's solution for several minutes the rate of the heart-beat is accelerated considerably. If then the Ca-free Ringer's solution is replaced by a normal Ringer's solution, the rate falls progressively to normal (rapidly at first and more gradually later), while the amplitude undergoes a somewhat rapid decrease to a minimum height followed by a period of gradual rise to normal (Fig. 5). This primary and temporary decrease in amplitude on change from a Ca-free to a normal Ringer's solution resembles that observed in the K-paradox, though it is less pronounced, and is known

FIG. 5. Experiment No. 26. (*a*) Ca-paradox obtained on change from a Ca-free to a normal Ringer's solution after the ganglion has been immersed in the former for twenty minutes. (*b*) Eighteen minutes later in the normal Ringer's solution.

as the Ca-paradox. Like the K-paradox, it is more readily obtained on a second or a third repetition than on the first immersion of a fresh ganglion in the Ca-free Ringer's solution; and the primary decrease in amplitude is greater after a longer period of immersion in the Ca-free Ringer's solution. The change illustrated in Fig. 5, for instance, was obtained after an immersion of a fresh ganglion in the Ca-free Ringer's solution for twenty minutes; a second immersion for ten minutes gave a very small primary decrease in amplitude indeed.

The Ca-paradox differs from the K-paradox essentially in two respects. In the first place, the primary temporary inhibition affects the amplitude only; during the process of recovery, the rate returns pro-

[6] The K-paradox can be more readily demonstrated on change from a K-free Ringer's solution to a sucrose solution containing KCl.

gressively to normal without first decreasing to a minimum. Since the amplitude is not always a good criterion for quantitative evaluation of the changes (*e.g.*, the amplitude is often markedly irregular in such a solution), and since the Ca-free Ringer's solution is more toxic than the K-free Ringer's solution, the Ca-paradox is not so easily obtained as the K-paradox. For this reason the observations on the Ca-paradox have been relatively few. In the second place, it seems that the Ca-paradox is obtained only on change from a Ca-free to a normal Ringer's solution. It is not obtained on change from a Ca-free Ringer's solution to a sucrose solution containing 37 millimols $CaCl_2$ in a liter, or to a K-free Ringer's solution.[7] Furthermore, the presence of an excess of $CaCl_2$ in the second solution prevents the appearance of any change of the paradoxical kind; there is simply a progressive decrease in rate and amplitude. In short, the Ca-paradox is not proportional to the difference in the Ca-content of the two solutions and requires the presence of both NaCl and KCl.

*The Thermo-paradox*

The rate of the heart-beat of *Limulus* is always increased at a higher temperature and decreased at a lower temperature. Therefore, on sud-

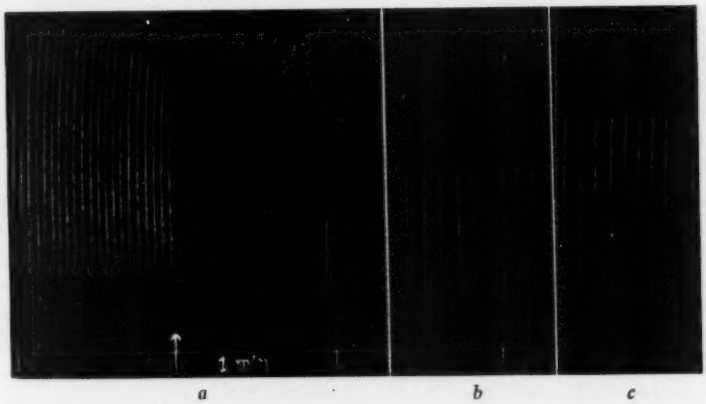


FIG. 6. Experiment No. 32. (*a*) Thermo-paradox obtained on change from a warm Ringer's solution at 41.5° C. to another Ringer's solution at room temperature (24.0° C.). (*b*) Seven minutes later; and (*c*) 14 minutes later at the room temperature.

denly bringing a ganglion from a Ringer's solution at the room temperature to a warm Ringer's solution, the rate is almost instantaneously in-

[7] The changes obtained on transfer from a pure NaCl solution to a normal Ringer's solution resemble the K-paradox rather than the Ca-paradox, for usually both the rate and the amplitude undergo the primary and temporary decrease.

creased. On bringing it back to the room temperature, the rate would be expected to return either rapidly, or gradually, to normal. What actually happens, however, is of the general nature designated as paradoxical—both the rate and the amplitude are immediately decreased to a level below normal, or the heart-beat may be completely arrested for a short period; then the normal rhythm for the room temperature is gradually resumed. This primary and temporary inhibition of the cardiac rhythm on change from a high to a low temperature is called the thermo-paradox. In general this effect is more pronounced the greater the decrease in temperature. When the temperature was suddenly decreased from 41.5° to 24.0° (Fig. 6), the heart-beat was immediately arrested for about half a minute and a gradual recovery followed. When the temperature was then decreased from 33.5° to 24.0° on the same ganglion, the heart-beat was greatly decreased both in rate and in amplitude, but not completely arrested, and the recovery was complete in a relatively short period.

## Discussion

The demonstration of K-paradox, Ca-paradox and thermo-paradox in the ganglion of the *Limulus* heart as well as in the vertebrate heart offers additional evidence of the general physiological similarity between these hearts. The paradoxes observed in the ganglion of the *Limulus* heart differ, however, from those of the vertebrate heart in that while in the former both rate and amplitude may be involved in the primary temporary inhibition, in the latter the primary inhibition affects the rate only. This difference is to be expected when one considers that the vertebrate heart is strictly of the all-or-none type; while in the *Limulus* heart the amplitude of the contractions may vary greatly, apparently in correlation with the number of nerve cells acting simultaneously in the ganglion. A decrease in amplitude means simply a decrease in the number of active nerve cells. With the exception of this apparent difference and a few minor points, the paradoxes found in the ganglion of the *Limulus* heart are essentially identical with those described for the vertebrate heart.

Many explanations have been suggested for the paradox phenomena. In connection with his work on physiological radioactivity Zwaardemaker (1917, 1921) attempted to explain his radio-physiologic paradoxes in terms of the antagonistic actions between the light and the heavy radioactive substances when applied successively. Libbrecht believed that the K-paradox is a specific K-effect connected with the ability of the tissue to adapt itself to changes in environmental conditions. In his paper on the rabbit's heart, Busquet pointed out certain resem-

blances between the K-paradox and the effects of vagus stimulation. Yet the K-paradox can still be produced after the action of the vagus nerve is abolished by atropine (Busquet, 1921, and Kisch, 1927). Witanowski (1926) regarded a change in the permeability of the cell membrane to K-ion in the K-free solution as the essential factor in the production of the K-paradox. But the Ca-paradox, Sr-paradox, and thermo-paradox cannot be satisfactorily explained on these theories; and the demonstration of these paradoxes in the ganglion of the *Limulus* heart and in other tissues with automatic rhythmic activity indicates that the phenomenon is a general one, and that some general, or unitary mechanism is involved. Two distinct and equally important factors appear to be concerned: (1) a preliminary process in a modified medium, and (2) a process connected with the sudden return to the normal medium. The paradox appears almost simultaneously with the change of the solutions, indicating apparently a surface phenomenon. The preliminary process (*e.g.*, the immersion in a K-free, or a Ca-free, or a warm Ringer's solution) is preparatory in nature and requires a minimum duration; its effect is to establish a new equilibrium in the tissue such that on subsequent return to its normal medium a primary and a purely temporary inhibition of activity occurs before the tissue can resume its normal condition, *i.e.*, during the temporary period of disequilibration in the second solution. This is the essence of the paradox phenomenon.

## Summary

Potassium-paradox, calcium-paradox, and thermo-paradox have been observed in the ganglion of the *Limulus* heart.

The Ca-paradox is obtained on change from a Ca-free to a normal Ringer's solution, and the thermo-paradox is obtained on change from a warm to a cold Ringer's solution.

The K-paradox is obtained in a similar way on change from a K-free to a normal Ringer's solution, and its production depends upon the following factors:

1. The K-paradox is more readily obtained on the second or third repetition than on the first immersion of a fresh ganglion in the K-free Ringer's solution.

2. The paradoxical effect becomes more pronounced with increasing duration of the preliminary immersion in the K-free Ringer's solution.

3. The factor determining the K-paradox is the sudden change in the K-content of the medium surrounding the ganglion, and this change must be in the direction of increasing the K-content in the second solution.

4. The presence of a minimum amount of NaCl in the K-free Ringer's solution (about 30 per cent of the normal concentration) is necessary for the production of the K-paradox. The presence of $CaCl_2$ in the K-free, or the K-containing, solution is of only secondary importance perhaps by virtue of its antagonistic action.

In general the paradoxes found in the ganglion of the *Limulus* heart are essentially identical with those described in the vertebrate heart.

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## REFERENCES

BAKKER, B., 1927. Onderzoekingen in het physiol. laborat. d. Rijksuniversiteit te Utrecht, **7**: 129.
BUSQUET, H., 1921. *Compt. rend. Soc. de Biol.*, **85**: 1142.
BUSQUET, H., 1922*a*. *Compt. rend. Soc. de Biol.*, **86**: 106.
BUSQUET, H., 1922*b*. *Compt. rend. Soc. de Biol.*, **86**: 1010.
CHAO, I., 1933. *Biol. Bull.*, **64**: 358.
HOBSON, A. D., 1928. *Brit. Jour. Exper. Biol.*, **5**: 385.
KISCH, B., 1927. *Arch. f. exper. Pathol. u. Pharmakol.*, **122**: 257.
KISCH, B., 1930. *Arch. f. exper. Pathol. u. Pharmakol.*, **148**: 140.
LIBBRECHT, W., 1920. *Arch. internat. de physiol.*, **15**: 446.
LIBBRECHT, W., 1921. *Arch. internat. de physiol.*, **16**: 448.
WELLS, G. P., 1928. *Brit. Jour. Exper. Biol.*, **5**: 258.
WITANOWSKI, W. R., 1926. *Pflüger's Arch.*, **212**: 726.
ZWAARDEMAKER, H., 1917. *K. Akad. v. Wetensch. Amsterdam*, **19**: 1043.
ZWAARDEMAKER, H., 1921. *Ergebn. d. Physiol.*, **19**: 326.
ZWAARDEMAKER, H., 1925. *Ergebn. d. Physiol.*, **24**: 166.

# HEMATOLOGICAL STUDIES IN DOGFISH (MUSTELUS CANIS) [1]

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## I. The Normal Blood Picture and the Effect of Removal of Blood and of Turpentine Injections

In the mammal the blood-forming organs, and therefore the blood-cell picture, respond rapidly to environmental changes. This is seen in infections (Goodner, 1928; Reznikoff, 1929), severe exercise (Garrey and Butler, 1929) and in many conditions involving nutritional disturbances (Keefer, 1932). Certain blood cells, for example the neutrophiles, vary in number and in degree of maturation markedly and very soon after the onset of hemorrhage or the inoculation of bacteria. Since fish lack those white blood cells which form the important class of polymorphonuclear leucocytes in the mammal, it was thought of interest to determine what cells take their place, if the same agents were used which provoke an outpouring of these cells in mammals. Incidentally, some standard hematological values might be obtained by using the ordinary quantitative clinical pathological methods of enumeration and estimation.

Dogfish (*Mustelus canis*) were selected for this study because of their availability and because they could be manipulated outside the water for a considerable time without any appreciable struggle involving asphyxia. Blood was removed from the heart in small amounts, 0.5 to 0.75 cc., with a syringe and needle which had previously been rinsed with 1 per cent aqueous heparin solution. This prevented clotting effectively without diluting the blood appreciably. Total red and white cell counts were made using Toison's solution as a diluent with a standardized pipette and in a Hellige improved Neubauer hemocytometer. Hemoglobin readings were made with a Sahli hemoglobinometer standardized to give 14.5 grams of hemoglobin per 100 cc. as 100 per cent. Differential counts were made by the supravital method following the technique of Sabin (1929).

In this study six fish were used for post-mortem study, no blood be-

[1] We wish to express our thanks to Dr. N. C. Foot and to Miss A. M. McDowell for their help in the pathological work of this study.

ing removed prior to their death. Twelve fish were used to determine the effect of frequent removal of blood from the heart upon the blood picture. Four fish were injected intraperitoneally with 1.5 cc. of sterile distilled water. Eight fish were injected intraperitoneally and one intramuscularly with turpentine in doses varying from 0.25 to 1.5 cc. Most of the injections were in 1 cc. amounts.

The initial counts in twenty-five fish gave the results shown in Table I.

TABLE I

*Blood-counts in Twenty-five Normal Dogfish*

| | Average | Range |
|---|---|---|
| Hemoglobin | 30 per cent (4.35 grams per 100 cc. of blood) | 19–41 per cent |
| Red blood cells | 393,000 per cu. mm. | 210,000–630,000 |
| White blood cells | 97,000 per cu. mm. | 60,000–178,000 |
| Differential: | | |
| Small lymphocytes | 54 per cent | 35–71 per cent |
| Large lymphocytes | 9 " " | 2–17 " " |
| Thrombocytes | 18 " " | 6–32 " " |
| Eosinophiles | 14 " " | 5–32 " " |
| Pseudoeosinophiles | 4 " " | 1–8 " " |
| Monocytes | 1 " " | 0–2 " " |

Removal of blood from the heart infrequently or in small amounts caused no change in the red cell count and hemoglobin percentage. In most cases, however, some reduction in the white blood cell count was obtained, usually slight to moderate. Practically no variation occurred in the differential count except that when blood was withdrawn shortly before a fish died, the thrombocytes increased in number and the eosinophiles decreased. This, however, is found as a rule in all fish before death regardless of the cause. An illustration of the results obtained in a case where only small amounts of blood were removed is shown in Table II.

On the other hand, much loss of blood results in a marked decrease in the red cell count and in the hemoglobin percentage. The white cells are markedly depressed without any striking change in the differential count. Table III illustrates such a case. From this fish much blood was taken for the first count and considerable oozing of blood occurred after the puncture.

Injection of 1.5 cc. of sterile distilled water intraperitoneally has apparently no effect upon the blood picture of the fish. They showed no changes other than those seen when blood is removed for successive counts or which occur just before death in any fish.

TABLE II

*The effect on the blood picture of removing small quantities of blood (less than 0.75 cc.).* Fish No. 28.

| Time of removal of blood | Hemoglobin | R.B.C. | W.B.C. | Differential count | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Lymphocytes | Large Lymph. | Thrombocytes | Eosinophiles | Pseudoeosin. | Monocytes |
| | *per cent* | *per cu. mm.* | *per cu. mm.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 8/16/33 9:00 A.M. | 30 | 364,000 | 80,000 | 53 | 11 | 17 | 17 | 2 | 0 |
| 8/17/33 9:00 A.M. | 32 | 349,000 | 65,000 | 39 | 16 | 24 | 18 | 2 | 1 |
| 8/18/33 9:00 A.M. | 33 | 392,000 | 68,000 | 52 | 7 | 26 | 14 | 1 | 0 |
| 8/19/33 9:00 A.M. | 30 | 393,000 | 76,000 | 55 | 3 | 30 | 10 | 2 | 0 |

TABLE III

*The effect on the blood picture of removing large quantities of blood (more than 0.75 cc.).* Fish No. 29.

| Time of removal of blood | Hemoglobin | R.B.C. | W.B.C. | Differential count | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Lymphocytes | Large lymph. | Thrombocytes | Eosinophiles | Pseudoeosin. | Monocytes |
| | *per cent* | *per cu. mm.* | *per cu. mm.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 8/16/33 9:30 A.M. | 30 | 344,000 | 104,000 | 64 | 6 | 16 | 11 | 2 | 1 |
| 8/17/33 9:30 A.M. | 18* | 190,000 | 19,000 | 50 | 14 | 26 | 10 | 0 | 0 |
| 8/18/33 9:30 A.M. | 24 | 272,000 | 52,000 | 54 | 10 | 21 | 13 | 2 | 0 |

* In Tables III and IV, heavy lines are drawn around numbers which show the striking variations due to the experimental procedure.

TABLE IV

*The effect on the blood picture of injecting turpentine intraperitoneally*

| Time of removal of blood | Hemoglobin | R.B.C. | W.B.C. | Differential count | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Lymphocytes | Large lymph. | Thrombocytes | Eosinophiles | Pseudoeosin. | Monocytes |
| | *per cent* | *per cu. mm.* | *per cu. mm.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| *Fish No. 12* | | | | | | | | | |
| 8/8/13 4 P.M. | 35 | 559,000 | 101,000 | 50 | 5 | 12 | 27 | 6 | 0 |
| 0.5 cc. turpentine intraperitoneally 4:45 P.M. | | | | | | | | | |
| 7:30 P.M. | 39 | 425,000 | 61,000 | 45 | 9 | 22 | 23 | 1 | 0 |
| 8/9/33 9 A.M. | [19] | [344,000] | [28,000] | [78] | 3 | [6] | [8] | 3 | 2 |
| 2 P.M. | 17 | 252,000 | 30,000 | 70 | 26 | 1 | 3 | 0 | 0 |
| Dead 2:45 P.M. | | | | | | | | | |
| *Fish No. 13* | | | | | | | | | |
| 8/8/33 8 P.M. | 30 | 217,000 | 63,000 | 37 | 4 | 27 | 23 | 8 | 1 |
| 1 cc. turpentine injected intraperitoneally 9:45 P.M. | | | | | | | | | |
| 8/9/33 9:10 P.M. | 32 | 416,000 | 70,000 | 65 | 5 | 24 | [3] | 2 | 1 |
| 6:45 P.M. | 20 | 207,000 | [25,000] | [79] | 6 | [6] | [5] | 3 | 1 |
| 8/10/33 9 A.M. | 33 | 321,000 | 47,000 | 67 | 5 | [24] | 2 | 1 | 1 |
| Dead 6 P.M. | | | | | | | | | |
| *Fish No. 15* | | | | | | | | | |
| 8/10/33 11:30 A.M. | 21 | 327,000 | 78,000 | 55 | 10 | 17 | 9 | 8 | 1 |
| 1 cc. turpentine intraperitoneally 12:20 P.M. | | | | | | | | | |
| 8:20 P.M. | 19 | 142,000 | [23,000] | 66 | 13 | [1] | 14 | 4 | 2 |
| 8/11/33 9 A.M. | 19 | 320,000 | 73,000 | 54 | 14 | 25 | [3] | 3 | 1 |
| 6:45 P.M. | 20 | 237,000 | 26,000 | [80] | 13 | [4] | [1] | 1 | 1 |
| Dead 7:30 P.M. | | | | | | | | | |

TABLE IV—*Continued*

| Time of removal of blood | Hemoglobin | R.B.C. | W.B.C. | Differential count | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Lymphocytes | Large lymph. | Thrombocytes | Eosinophiles | Pseudoeosin. | Monocytes |
| | *per cent* | *per cu. mm.* | *per cu. mm.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| *Fish No. 16* | | | | | | | | | |
| 8/10/33 8 P.M. | 36 | 359,000 | 127,000 | 37 | 9 | 20 | 32 | 1 | 1 |
| 1 cc. turpentine intraperitoneally 8:50 P.M. | | | | | | | | | |
| 8/11/33 9:15 A.M. | 32 | 393,000 | [70,000] | [79] | 3 | [13] | [4] | 1 | 0 |
| 7:30 P.M. | 23 | 308,000 | 71,000 | 71 | 9 | 12 | 5 | 3 | 0 |
| 8/12/33 9 A.M. | 33 | 432,000 | 75,000 | 80 | 4 | 11 | 4 | 1 | 0 |
| Dead 12 M. | | | | | | | | | |
| *Fish No. 18* | | | | | | | | | |
| 8/14,33 9 A.M. | 32 | 442,000 | 178,000 | 69 | 8 | 6 | 15 | 1 | 1 |
| 0.25 cc. turpentine intraperitoneally 10:45 A.M. | | | | | | | | | |
| 8/15/33 9 A.M. | 23 | 319,000 | [38,000] | 70 | 2 | [21] | [4] | 3 | 0 |
| Dead 8/16/33 A.M. | | | | | | | | | |
| *Fish No. 19* | | | | | | | | | |
| 8/14/33 9:10 A.M. | 41 | 423,000 | 146,000 | 51 | 15 | 19 | 13 | 2 | 0 |
| 0.25 cc. turpentine intraperitoneally 10:50 P.M. | | | | | | | | | |
| 8/15/33 9:10 A.M. | 36 | 333,000 | [87,000] | [66] | 8 | 20 | [3] | 2 | 1 |
| Dead 8/16/33 A.M. | | | | | | | | | |

When turpentine is injected intraperitoneally in fish in amounts mentioned previously, very definite changes occur which cannot be explained on the basis of the control studies (removal of blood, injection of distilled water). Invariably a distinct leukopenia ensues and the differential picture shows a marked increase in the percentage of lymphocytes, an immediate and persistent decrease of eosinophiles and usually an initial decrease of thrombocytes which in some fish may increase just before death and in others remain low. The difference between this picture and the usual ante-mortem differential count is that many of the fish which received injections of turpentine showed blood changes long before they died and while they were in excellent condition. This healthy state can be judged by activity and the color of their skin, which shortly before death, or if the fish is in poor condition, loses its dark color due to contraction of the chromatophores.

Table IV shows the results obtained with some of the fish which were injected with turpentine intraperitoneally.

TABLE V

*Post-mortem Differential Counts on Fish*

| | Lympho-cytes | Large lympho-cytes | Thrombo-cytes | Eosino-philes | Pseudo-eosino-philes | Mono-cytes |
|---|---|---|---|---|---|---|
| | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| Fish No. 28 | 75 | 1 | 18 | 1 | 1 | 0 |
| Fish No. 31 | 77 | 4 | 17 | 1 | 0 | 1 |

The results of examination of the intraperitoneal exudate before as well as after death showed a tremendous outpouring of lymphocytes, small, intermediate, and large, many of them full of phagocytized granules, globules, probably of turpentine, and bacteria. In some cases the exudate had the appearance of actual pus. One of the surprising findings was that bacteria and exudate were present in fish which died "naturally," that is, from which no blood was withdrawn and in which no needle had been inserted into the peritoneal cavity before death. In one control fish which jumped out of the tank and was found dead the next morning, no exudate and bacteria were found in the peritoneum.

Some of the fish showed bacteria in the blood just before death and in the post-mortem blood.

In two of the fish post-mortem differential blood smears showed a picture which is illustrated by Table V.

The high lymphocyte count, the moderate number of thrombocytes, and the almost complete repression of eosinophiles in the post-mortem blood emphasizes further the trend toward such a blood picture as death approaches.

It is, of course, important to see if the change in the blood picture due to impending death or due to the injection of a substance like turpentine was a reflection of the condition of the spleen, the hematopoietic organ of the fish. Supravital smears from freshly cut surfaces of the spleens of these fish immediately after death showed in the fish injected with turpentine an almost complete absence of eosinophiles. Smears from the spleens of normal fish showed a considerable number of eosinophiles. Fixed tissue studies demonstrated that in the injected fish, compared to controls, the spleens either had a marked diminution of eosinophiles or that these cells were not compact and the eosinophilic granules were swollen and took a faint dull pink stain. There was no question but that there was a distinct hyperplasia of the lymphocytic elements which showed hyperchromatic characters in the injected fish.

## Discussion

Many features of these studies merit discussion. It is significant, for example, to note that the rather high eosinophile count in these dogfish under normal conditions may be associated with the prevalence of parasitic infestation (Stunkard, 1933).

In comparing the blood picture of the dogfish with that of the mammal under similar conditions of treatment, it is evident that the lymphocyte takes the place of the mammalian neutrophilic granulocyte or polymorphonuclear cell as far as its response to injury, its phagocytic ability, and its ameboid properties are concerned. Moreover, it is apparent that the so-called granulocyte is really a large lymphocyte, as suggested by Jordan and Speidel (1924), since all transition stages can be traced between the two. Perhaps the most interesting phase of this study is the persistent leukopenia seen also at the beginning of infections in mammals and sometimes extending throughout the course of disease. In the dogfish apparently this leukopenic stage does not pass over into leukocytosis. The increased production of lymphocytes as evidenced by hyperplasia of the spleen results in a concentration of these cells at the site of irritation,—in this study, the peritoneal cavity. In mammals Ewing (1895) and Doan and his coworkers (1928) found a marked increase of cells in the spleen and liver in the early stages of infection. But as the disease progressed the blood reflected the increase of cell production in most cases by showing a marked increase in cells. It would be of interest to determine where in the phylogenetic scale this ability to change from a leukopenia to a leukocytosis occurs. In a disease known as acute glandular fever in humans an excessive response of lymphocytes, as in these fish, is also seen. These problems may have considerable interest to the comparative pathologist.

## Conclusions

1. The blood picture in twenty-five dogfish (*Mustelus canis*) has been studied and the findings recorded.

2. Frequent removal of blood or removal of blood in large amounts from fish may cause an anemia and a leukopenia but produces no change in the relative numbers of the different white blood cells.

3. Injection of sterile distilled water intraperitoneally causes no change in the blood picture.

4. Injection of turpentine intraperitoneally causes a distinct leukopenia, a relative increase of lymphocytes, usually a decrease of thrombocytes and always a marked decrease of eosinophiles.

5. Just before death control fish, as well as those which had been injected with turpentine, may show an increase of thrombocytes.

6. Studies of the peritoneal exudate and the spleen, as well as of the blood, indicate that turpentine acts as a definite chemotactic agent stimulating production of lymphocytes. The lymphocyte is the actively responding cell to acute irritation in the dogfish and has the same significance as the neutrophile (polymorphonuclear) in mammals.

7. Antemortem and postmortem studies suggest that bacterial invasion of the blood stream and of the peritoneal cavity is not uncommon even in control fish and that blood changes (increase of lymphocytes and decrease of eosinophiles) may occur just before death.

8. This investigation indicates that the changes observed may be of importance in comparative biological problems.

## BIBLIOGRAPHY

Doan, C. A., L. G. Zerfas, S. Warren, and O. Ames, 1928. A Study of the Mechanism of Nucleinate-induced Leucopenic and Leucocytic States, with Special Reference to the Relative Rôles of Liver, Spleen and Bone Marrow. *Jour. Exper. Med.*, **47**: 403.

Ewing, J., 1895. Toxic Hypoleucocytosis. *N. Y. Med. Jour.*, **61**: 257.

Garrey, W. E., and V. Butler, 1929. Physiological Leukocytosis. *Am. Jour. Physiol.*, **90**: 355.

Goodner, K., 1928. Experimental Intradermal Pneumococcus Infection in Rabbits. *Jour. Exper. Med.*, **48**: 1.

Jordan, H. E., and C. C. Speidel, 1924. Studies on Lymphocytes. II. The origin, function and fate of the lymphocytes in fishes. *Jour. Morph.*, **38**: 529.

Keefer, C. S., 1932. Some Clinical Features of Deficiency Disorders. *N. Y. State Jour. Med.*, **32**: 1405.

Reznikoff, P., 1929. Immature White Blood Cell Counts in Infectious Diseases. *Jour. Am. Med. Ass'n*, **93**: 963.

Sabin, F. R., 1929. Supravital Stains, Microscopical Technique. Edited by McClung. Paul B. Hoeber, Inc., N. Y.

Stunkard, H. W., 1933. Personal Communication.

# FURTHER OBSERVATIONS ON THE SALT REQUIREMENTS OF LIGIA IN BERMUDA [1]

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A previous paper (Barnes, 1932) dealt with the salt requirements and space orientation of the littoral isopod *Ligia baudiniana* in Bermuda. It was shown that *Ligia* is unable to withstand prolonged immersion in sea water and survives best in moist air. The isopod is poikilosmotic and dies within a few hours in distilled water, fresh water, and sea water of half dilution or less. Its survival in natural sea water is not much longer than in artificial sea water even if the latter lacks Mg. Experiments with 5/8 M solutions of single salts established the toxicity series K > Mg >Ca > Na.

The present paper describes the longevity of *Ligia* in weaker solutions of single salts, in antagonistic solutions, and when living in air with access to salt solutions.

## HABITS

Although *Ligia* occurs in great numbers on most of the rocky shores of the islands, we have very rarely observed the animal in the act of feeding. Nicholls (1930–31) describes the nocturnal "browsing" of *L. oceanica,* which clings to *Fucus* and cuts off very small portions with its mandibles. During the summer of 1933 it was found that the feeding of *L. baudiniana* can be easily watched if the observer remains motionless on the rocks when the tide begins to recede. The isopods leave their hiding places and follow the ebbing tide, feeding on the algal covering of the damp rocks. Unlike *L. oceanica, L. baudiniana* appears during the day and is positively phototropic in the laboratory. After the intertidal zone is dried by the sun, they usually retreat under stones and in crevices but some remain near the water's edge. The animal scrapes off the unicellular algæ by a vertical motion of the head, the whole anterior half of the body taking part in the rasping movement. The animal feeds for a short interval and moves on for about 10 cm. and feeds again; this intermittent process is especially evident when the intertidal zone is first invaded.

In the preceding paper it was shown that *Ligia* must keep its gills

[1] Contribution from the Bermuda Biological Station for Research.

moist for aerial respiration. Water will rise by capillarity up the spines and uropods or the gills may be brought into contact with a moist substratum by lowering the abdomen. This reflex is elicited by contact with sea water on any part of the body. If a drop of sea water is placed

TABLE I

*Longevity of Ligia in Air and in Water*

| Medium | Year | Average longevity | Maximum longevity | No. specimens tested |
|---|---|---|---|---|
| | | *hours* | *hours* | |
| Distilled water | 1932 | 4 | 5 | 21 |
| | 1933 | 3.9 | 5 | 33 |
| | Both years | 4 | 5 | 54 |
| Fresh water | 1932 | 7½ | 8 | 21 |
| | 1933 | 7 | 9 | 9 |
| | Both years | 7 | 9 | 30 |
| Sea water | 1932 | 34 | 297 | 93 |
| | 1933 | 74 | 240 | 25 |
| | Both years | 42½ | 297 | 118 |
| 2.5 cc. sea water plus 100 cc. $H_2O$ | 1933 | 4½ | 6 | 10 |
| 25% sea water | 1932 | 6 | 10 | 6 |
| | 1933 | 6½ | 7½ | 24 |
| | Both years | 6½ | 10 | 30 |
| 50% sea water | 1932 | 20 | 70 | 7 |
| | 1933 | 17½ | 64 | 15 |
| | Both years | 18 | 70 | 22 |
| Air (dry bowls) | 1932 | 11 | 12 | 8 |
| | 1933 | 17¾ | 25 | 49 |
| | Both years | 15¼ | 25 | 57 |
| Air over damp sand | 1932 | 360 | 625 | 31 |
| | 1933 | 384 | 648 | 51 |
| | Both years | 375 | 648 | 82 |

on the head of an animal which has been out of water some time, the uropods are first brought together and then lowered to the substratum, even if the latter is dry. This reflex is so strong that a running animal

drags its spines when traversing a wet region. It is probable that *Ligia baudiniana* is kept moist by its habit of hiding in crevices or under fragments of the porous Bermuda rock which is saturated at high tide. Many specimens are so moist that they glisten in the sun, but I have never found submerged specimens. A pursued isopod will enter the water only if no other path of escape is available but soon returns to air. It is interesting to observe the mass migrations to upper regions of the shore as the tide advances. The individuals at the water's edge are the first to start landwards, but these are joined by specimens several feet from the water line. Individuals of all sizes exhibit this periodic retreat, the mature ones being in advance and always ascending the furthest inland. In captivity the young are released only by females kept in bowls of sea water, but the young (usually about eighty from one specimen) immediately crawl out of the water if any available projection is present. During the past summer the isopods were collected from the intertidal zone at low tide by shaking loose stones over a pail with smooth vertical sides. The specimens dealt with in the previous paper were taken on a wall several feet from the sea. This fact may help to explain differences in the longevity data for the two series (see Table I).

The rôle of positive geotropism as an important factor in the orientation of *Ligia* towards the sea when released some distance inland was confirmed and the tendency of half-grown specimens to crawl down slopes was again observed. In one experiment the isopods crawled toward the sea *up* a slight incline (sand) of 10°, but on a 30° slope in the same region they crawled down *away* from the sea. The orientation of the isopod to shore when released in the sea was studied in tidal pools. Isopods released in the center of a pool swim or crawl to the water's edge in all radial directions regardless of the direction of the mainland. In the water their slow swimming movements make them an easy prey to shore fishes but their rapidity on land enables them to escape pursuit by the mangrove crab *Goniopsis cruentatus*. However, *Ligia* does not occur in mangrove swamps where *Goniopsis* is very abundant.

### Longevity of Ligia in Air and in Water

The number of individuals tested in some of the 1932 experiments was not large and it was necessary to repeat some of the tests, especially since the specimens were taken from a habitat nearer the water line. The same average longevity was found in distilled water,—4 hours,—and in fresh water, 7 hours, but the 1933 isopods lived longer in sea water (Table I). However, we regard the average of the two series, 42 hours, as a satisfactory working average for comparison with other

media. The 1933 series of isopods in dry dishes lived an average of 17¾ hours, 6¾ hours longer than the 1932 series. This was probably due to the fact that the 1932 isopods were collected several feet above the high water line and had been exposed to desiccation. In air over damp sand the new series gave a slightly greater longevity, 384 as compared to 360 hours. Moulting was observed only in these specimens and not in submerged ones. The duration of life in 25 per cent and 50 per cent sea water, 6 and 18 hours respectively, was nearly the same in each series. In general it will be seen that the same relative effect was secured in the various media in spite of the difference in year, number of specimens, habitat, and method of collecting. In both series individual specimens were tested in bowls containing 100 cc. of solution.

## Toxicity of Single Salts

The survival of *Ligia* was determined in distilled water containing the percentage of a single salt occurring in sea water for comparison

Table II

*Longevity of Ligia Immersed in Solutions of Single Salts*

| Medium | Average longevity | Maximum longevity | No. specimens |
|---|---|---|---|
| | *hours* | *hours* | |
| 5/8 M NaCl 1933 | 8 | 9 | 18 |
| 5/8 M NaCl 1932 | 8 | 14 | 12 |
| Both years | 8 | 14 | 30 |
| 2.5 cc. 5/8 M NaCl plus 100 cc. $H_2O$ | 2¾ | 3½ | 10 |
| 2.5 cc. 5/8 M $CaCl_2$ plus 100 cc. $H_2O$ | 9¼ | 11 | 30 |
| 2.2 cc. 5/8 M KCl plus 100 cc. $H_2O$ | 3 | 4 | 27 |
| 50 cc. 5/8 M KCl plus 100 cc. $H_2O$ | 2¼ | 2½ | 10 |
| 11.6 cc. 5/8 M $MgCl_2$ plus 100 cc. $H_2O$ | 7 | 9 | 13 |

with the toxicity series of 5/8 M single salts previously described. In a solution containing 2.5 cc. of 5/8 M NaCl plus 100 cc. of water the average duration of life was only 2¾ hours with a maximum of 3½ hours, while in $CaCl_2$ of the same concentration the average longevity was 9¼ hours with a maximum of 11 hours (Table II). The results are of interest since NaCl is the least toxic salt in solutions isosmotic with Bermuda sea water. A 5/8 M NaCl series was repeated and the same average longevity, 8 hours, obtained as previously, showing that the 1933 specimens did not differ in their reaction to sodium chloride. In 2.2 cc. of 5/8 M KCl plus 100 cc. of water, *Ligia* lived an average of 3 hours with a maximum of 4 hours, and durations of approximately the same order were found for 50 cc. 5/8 M KCl plus 100 cc. water, *i.e.*,

TABLE III

*Longevity of Ligia in Antagonistic Solutions*

| Solution | Average longevity | Maximum longevity | No. of specimens tested |
|---|---|---|---|
| | *hours* | *hours* | |
| Artificial sea water (1932) | 40 | 123 | 8 |
| 5/8 M artificial sea water lacking NaCl (1932) | 2 | 2 | 5 |
| Same (1933) | 2¾ | 3¼ | 12 |
| Artificial sea water lacking NaCl (NaCl replaced by distilled water) | 5¾ | 9 | 20 |
| 5/8 M artificial sea water lacking NaCl and $MgCl_2$ | 1¾ | 2¼ | 10 |
| Artificial sea water lacking Na and Mg (NaCl and $MgCl_2$ replaced by distilled water) | 9½ | 12 | 17 |
| 1 part 5/8 M NaCl plus 1 part 5/8 M $CaCl_2$ | 24¾ | 28 | 4 |
| 4 parts 5/8 M NaCl plus 1 part 5/8 M $CaCl_2$ | 51¼ | 96 | 15 |
| 2.5 cc. 5/8 M $CaCl_2$ plus 100 cc. 5/8 M NaCl | 21 | 40 | 20 |
| 1 part 5/8 M $CaCl_2$ plus 1 part 5/8 M KCl | 4 | 6 | 10 |
| 2.2 cc. 5/8 M KCl plus 100 cc. 5/8 M NaCl | 10½ | 14 | 10 |
| 4.4 cc. 5/8 M KCl plus 100 cc. 5/8 M NaCl | 6½ | 9 | 10 |
| 11.6 cc. 5/8 M $MgCl_2$ plus 100 cc. 5/8 M NaCl | 9¼ | 18 | 20 |

average 2¼ hours, maximum 2½ hours. It was reported in the first paper that *Ligia* lives an average of 1½ hours in 5/8 M KCl in which the gills are completely paralysed. In the weak KCl solution, however. the gills vibrated normally but were stopped in the solution containing

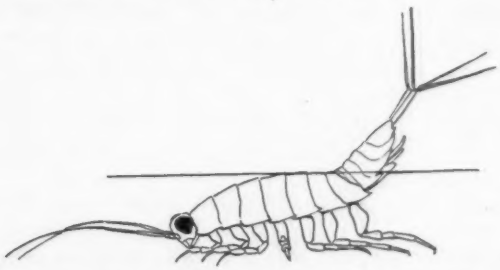

FIG. 1. A specimen in a solution of equal parts NaCl and KCl 5/8 M in which the gill beat is inhibited by the K. The animal has elevated its gills out of the water for aerial respiration.

50 cc. of 5/8 M KCl plus 100 cc. of water. In a solution containing the percentage of Mg in sea water (11.6 cc. of 5/8 M $MgCl_2$ plus 100 cc. of distilled water), the average longevity was 7 hours and the maximum 9 hours. It is clear that the toxicity series in weak solutions is quite different from that in solutions isosmotic with Bermuda sea water.

### Ionic Antagonism

In the first paper it was mentioned that no satisfactory binary mixture was found but antagonism seemed evident between Na and Ca. Further work with salt mixtures supports this statement (*vide* Table III). The most favorable of the binary solutions tested was composed of 80 per cent NaCl and 20 per cent $CaCl_2$ in 5/8 M concentration.

It was of interest to observe that the gills vibrated in the weak KCl solutions, thus showing the toxicity of the potassium ion apart from its inhibition of the gill beat, which is completely inhibited in 5/8 M KCl made in sea water, 5/16 M KCl, and in equal parts of 5/8 M KCl and NaCl. On the other hand, the gill rhythm occurs in the following solutions: 2.2 cc. 5/8 M KCl plus 100 cc. $H_2O$; 2.2 cc. 5/8 M KCl plus

Table IV

*Longevity of Ligia in Dry Bowls Containing Watchglasses of Various Solutions*

| Solution in watchglass | Average longevity | Maximum longevity | No. specimens tested |
|---|---|---|---|
| | *hours* | *hours* | |
| Sea water | 292¾ | 432 | 13 |
| Distilled water | 156 | 240 | 19 |
| 25% sea water | 240 | 432 | 8 |
| 1 part 5/8 M NaCl plus 1 part 5/8 M KCl | 10 | 19 | 14 |
| 5/8 M NaCl | 60 | 144 | 16 |
| 5/8 M $CaCl_2$ | 29 | 52 | 22 |
| 2.5 cc. 5/8 M $CaCl_2$ plus 100 cc. $H_2O$ | 156 | 216 | 18 |
| 5/8 M KCl | 8¼ | 24 | 24 |
| 2.2 cc. 5/8 M KCl plus 100 cc. $H_2O$ | 168 | 216 | 10 |

100 cc. 5/8 M NaCl; 4.4 cc. 5/8 M KCl plus 100 cc. 5/8 M NaCl; and in M/4 KCl made in sea water. In solutions inhibiting the gill beat the isopod sometimes elevates its moist gills out of the water for aerial respiration. (Fig. 1.)

### Longevity of Ligia Having Access to Salt Solutions

Watchglasses containing sea water or other solutions were placed in dry bowls containing an isopod (Table IV). In the less toxic solutions the isopod remained at the edge of the watchglass. This was observed with sea water, 5/8 M NaCl, 5/8 M $CaCl_2$, 2.5 cc. of 5/8 M $CaCl_2$ plus 100 cc. of water and 2.2 cc. of 5/8 M KCl plus 100 cc. of water. When the watchglass contained sea water, the isopod crawled through the solution so often that after a few days most of the sea water was in the bowl. In such cases the animal remained in the watchglass if this contained less water than the bowl.

As will be seen in Table IV, the animals lived for long periods with access to sea water, even when diluted to 25 per cent, and to distilled water. It was again possible to demonstrate the toxicity of KCl apart from its paralyzing action on the beating of the pleopods in submerged specimens.

## Discussion

It is of interest to compare the survival of *Ligia baudiniana* in hypotonic solutions with that of *L. oceanica* which appears to be somewhat less terrestrial since it sometimes remains covered by the tide. According to Tait (1916–17) and Nicholls (1930–31), *L. oceanica* survives for very long periods when kept in sea water. Its longevity in diluted sea water is also greater than that of *L. baudiniana*, Tait (1916–17) reporting a survival of as long as forty days in 50 per cent sea water and ten days in 25 per cent sea water (compare with Table I). However, both forms soon succumb in distilled water, the maximum being 5 hours for *L. baudiniana* and 36 for *L. oceanica*. Bateman (1933) finds that the blood of *L. oceanica* is approximately equal in concentration to sea water and that the isopod shows high osmotic independence for a short time when immersed in hypotonic sea water. Animals living on moist sea weed had a salt concentration of 0.631 M, rather greater than sea water. Nicholls (1930–31) kept *L. oceanica* for more than 15 months in moist air with *Fucus*. He reports a more rapid flapping of the pleopods in hypotonic sea water. In one instance 2 liters of sea water containing an isopod were diluted by 25 cc. each day. The time for ten beats decreased from 6 seconds on the fourteenth day to 5 seconds in a month's time. The frequency of gill rhythm of *L. baudiniana* also increases in diluted sea water. The time for ten beats decreased from 3 to 2.7 seconds in a sample specimen when transferred to 50 per cent sea water. Bateman (1933) found that the osmotic pressure of the blood in *oceanica* remained almost normal for several hours in this dilution but after 19 hours the osmoregulation broke down. The increased gill frequency can be interpreted in terms of Schlieper's (1929) theory, as a means of providing the extra oxygen necessary for work performed in the temporary resistance to osmotic forces. The investigations of Fox and Simmonds (1933), for example, show a higher oxygen consumption in species of *Gammarus* inhabiting fresh water.

According to Bogucki (1932), the marine isopod *Mesidotea entomon* exhibits considerable osmoregulation in hypotonic sea water and survives a few days in fresh water and 20 hours in distilled water. It contrasts rather sharply with *Ligia baudiniana*, which has less in common with fresh water animals.

The toxicity series for single salts isosmotic with Bermuda sea water is appreciably different from that for hypotonic solutions of the chief cations. The 5/8 M toxicity series K > Mg > Ca > Na becomes, in the weak solutions, Na > K > Mg > Ca. In the hypotonic solutions permeability is an important factor governing the loss of salts from the blood and Na, the least toxic ion in isosmotic concentration, becomes the most toxic, probably in its action of increasing permeability. In the same way Ca prevents the leaching action of water as Pantin (1931) has described in the estuarine worm *Gunda*. A similar effect has been described by McCutcheon and Lucké (1928) on sea urchin eggs and indeed was shown years ago by Osterhout (1915) in plant cells. *Ligia baudiniana* lives twice as long in 2.5 cc. of 5/8 M Ca plus 100 cc. water as in 2.5 per cent sea water (Tables I and II).

In binary mixtures with Na in natural proportions the Ca solution is the most favorable, but it is interesting to note a greater longevity when the Ca is increased to 20 per cent. Ca, however, does not antagonize the toxicity of K to a great extent (Table III) and in Na-K mixtures the longevity appears to be determined by the amount of K present.

The watchglass experiments showed the same toxicity series for solutions isosmotic with Bermuda sea water as in the immersion tests. With access to weak solutions, however, the isopods exhibited the same longevity with K and Ca. A mixture of equal parts of Na and K showed little antagonism in 5/8 M concentration, the average duration of life being one-thirtieth of that observed when the animals had access to sea water. The amphibious habits of *Ligia* render the isopod particularly suited for the study of specific ion effects which can be determined apart from osmotic and permeability disturbances.

## Summary

1. The toxicity series for the chief cations in sea water for *Ligia baudiniana* changes with the concentration of the single salts. In a weak solution Na is the most toxic but in 5/8 M concentrations it is the least toxic ion.

2. It is suggested that this is due to permeability effects in hypotonic solutions.

3. Na and Ca gave the most favorable binary mixture.

4. When kept in air with access to solutions of single salts *Ligia* shows the same toxicity series with respect to K, Na, and Ca as when immersed in isosmotic solutions.

I am indebted to Miss A. K. C. Barnes for valuable assistance with the experiments.

It is a pleasure to express my gratitude to Dr. J. F. G. Wheeler, who placed the facilities of the Bermuda Station at my disposal.

## CITATIONS [2]

BARNES, T. C., 1932. Salt Requirements and Space Orientation of the Littoral Isopod Ligia in Bermuda. *Biol. Bull.*, **63**: 496.

BATEMAN, J. B., 1933. Osmotic and Ionic Regulation in the Shore Crab, Carcinus mænas, with Notes on the Blood Concentrations of Gammarus locusta and Ligia oceanica. *Jour. Exper. Biol.*, **10**: 355.

BOGUCKI, M., 1932. Recherches sur la régulation osmotique chez l'isopode marin. Mesidotea entomon L. *Archiv. int. Physiol.*, **35**: 197.

FOX, H. M., AND B. J. SIMMONDS, 1933. Metabolic Rates of Aquatic Arthropods from Different Habitats. *Jour. Exper. Biol.*, **10**: 67.

MCCUTCHEON, M., AND B. LUCKÉ, 1928. The Effect of Certain Electrolytes and Non-electrolytes on Permeability of Living Cells to Water. *Jour. Gen. Physiol.*, **12**: 129.

NICHOLLS, A. G., 1930–31. Studies on Ligia oceanica. I. A. Habitat and Effect of Change of Environment on Respiration. B. Observations on Moulting and Breeding. *Jour. Mar. Biol. Ass'n.*, N. S., **17**: 655.

OSTERHOUT, W. J. V., 1915. On the Decrease of Permeability Due to Certain Bivalent Kations. *Bot. Gaz.*, **59**: 317.

PANTIN, C. F. A., 1931. The Adaptation of Gunda ulvae to Salinity. III. The electrolyte exchange. *Jour. Exper. Biol.*, **8**: 82.

SCHLIEPER, C., 1929. Über die Einwirkung niederer Salzkonzentration auf marine Organismen. *Zeitschr. f. vergl. Physiol.*, **9**: 478.

TAIT, J., 1916–17. Experiments and Observations on Crustacea. Part I. Immersion Experiments on Ligia. *Proc. Roy. Soc. Edin.*, **37**: 50.

[2] For additional references, see Barnes, 1932.

# THE CONCENTRATIONS OF PROTEASES, AMYLASE, AND LIPASE IN CERTAIN MARINE FISHES

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Marine fishes show great diversity as to food, feeding habits, special habitat, and rate and type of metabolism. Some have predominantly a fatty metabolism while others have essentially a carbohydrate metabolism in the sense that the nutritive reserves are stored in the form of fat or carbohydrates. Certain fishes, such as the mackerel, are extremely active; others are very sluggish; still others represent all the intergrades. Great variation is shown as to the development of the different organs concerned with digestion; in some fishes the pancreas is more or less clearly defined, in others it is diffuse and may be partially included in the liver. Pyloric cæca, which are the embryological equivalent of the exocrine pancreas, may also be its physiological equivalent in some cases. These structures are absent in many fishes.

As these factors vary, it is to be expected that there will be adaptive variations in the amounts and sites of production of the different digestive enzymes secreted. It is the purpose of this investigation to determine where the enzymes are secreted, and in what quantities, with a view to making correlations with the anatomy and physiology of the fishes studied.

The early literature bearing upon digestion in fishes is well reviewed by Yung (1899) and Sullivan (1907).

The rate of digestion has been studied by Van Slyke and White (1911). They state that in the dogfish two to three days are required for the disposal of a meal; this is about six times as long as the period in mammals, which may be due to the temperature difference. Svolima (1919) found the period of digestion to last up to five days. Yung (1899) had set the period at ten hours.

Kenyon (1925) compared digestion in the different classes of vertebrates. Peptic digestion he found to be remarkably uniform among all the animals investigated, with the exception of the stomachless carp. Gastric acidity is exceedingly variable, apparently depending upon the functional state of the organ. There seems to be no correlation between peptic activity and food habits. There is a close correlation between the

structure of the tract and the distribution of the enzymes. Amylase and invertase are present in herbivorous fishes to a larger extent than in carnivorous. Apparently there has been little change in the character of the enzymes or their rates of activity in the evolution of the amphibians, reptiles, and mammals from primitive types.

The production of relatively large amounts of carbohydrases in the herbivorous carp, as contrasted with the carnivorous pike, has also been pointed out by Vonk (1928).

It is generally agreed that the enzymes of fishes are similar to those of warm-blooded animals. The subject is reviewed by Chesley (1934) in a paper dealing with the temperature coefficients of amylases of different origins. It was found that the optimal temperatures (obtained under comparable, rigidly controlled conditions) were somewhat lower for the amylases of cold-blooded animals. The order found for the optimal temperatures is human amylase > terrapin amylase > fish amylase. Also it was found that fish amylase is relatively much more effective at 3.5° C. than either of the other two; human amylase is intermediate.

This latter finding does not accord with Riddle's (1909) general conclusion that "higher forms" progressively lose the power of digestion at lower temperatures. Riddle studied peptic digestion in Mett's tubes introduced into the stomachs of living animals kept in constant temperature baths. He also concluded that there is a progressive loss in digestive power in successively higher forms.

## Material

Fishes used in this study were selected as representatives of diverse specializations, as indicated in the introduction.

(1) Very active:
The mackerel, *Scomber scombrus* Linnæus.
The menhaden, *Brevoörtia tyrannus* (Latrobe).
The scup, *Stenotomus chrysops* (Linnæus).
(2) Intermediately active:
The sea robin, *Prionotus carolinus* (Linnæus).
The puffer, *Spheroides maculatus* (Bloch and Schneider).
(3) Inactive or sluggish:
The toadfish, *Opsanus tau* (Linnæus).

The mackerel, menhaden and scup represent active fishes, as shown by their rapid swimming, stream-line bodies, high blood sugars, high hemoglobin and blood iron concentrations and respiratory rates. The

menhaden differs from the other two in storing much of its reserve as fat. The mackerel and menhaden feed by swimming rapidly near the surface, and straining their food out of the water. The mackerel is carnivorous, and exercises a degree of selection for *Calanus* (Bigelow and Welsh, 1924). The menhaden is predominantly herbivorous, feeding upon diatoms and peridineans (Bigelow and Welsh, 1924; Peck, 1894).

The toadfish is very sluggish; it lies at the bottom waiting for prey, and does little active hunting. The body is depressed; the blood sugar, hemoglobin and iron are all low. The respiratory rate is correspondingly low.

The sea robin and puffer are of intermediate activity as judged by these criteria (Hall, 1929; Hall and Gray, 1929; Gray and Hall, 1930). The sea robin stores a large amount of fat, and for this reason may be classified with the menhaden in type of metabolism.

## Methods

### *Preparation of the Enzyme Solutions*

The fishes used in this investigation were kept in live cars or tanks for a few days before using, in order to have them in approximately the same condition as regards gastro-intestinal contents. With the mackerel this scheme was impracticable, and fresh fish had to be used whenever available. The body-weights of all the fishes fell between 200 and 280 grams.

The viscera were separated, washed, slit open, washed again, blotted, and weighed. The tissues were ground vigorously with glass fragments. In many cases the isolation of the pancreas was a physical impossibility; here the procedure was simply to grind up the mesentery, after removing portions which obviously did not contain pancreatic tissue. Concordant values in enzyme quantities reckoned as units per gram of "pancreatic tissue" are hardly to be expected.

Bile was usually collected from the gall bladder by means of a needle and syringe. In many experiments, the gall bladder was stripped of its serosa and ground up in the bile, and the whole extracted.

The extracting agent was added to the tissue in the proportion of 20 ml. per gram of tissue. The whole was put into a small flask and allowed to stand at room temperature for twenty-four hours. Fifty per cent alcohol or 50 per cent glycerine were used exclusively after it had been established that these were the most efficacious of the solutions tried. The gastric mucosæ were extracted with N/10 HCl.

The several organs were separately and simultaneously extracted.

### *The Measurement of Digestion*

*Pepsin and Trypsin.*—These proteases were estimated by the viscometric method described by Northrop and Hussey (1923). For pepsin, a 3 per cent gelatin preparation was used. The pH was maintained at 2.5. For trypsin, a similar gelatin solution was buffered at pH 7.4, using 0.05 M phosphate. The substrate (10 ml.) was put into an Ostwald viscosimeter in a water bath at 35° C.; 0.2 to 0.4 ml. of tissue extract was added when the gelatin had come to the temperature of the bath. Boiled enzyme was added to a similar system which served as a control. At intervals, the viscosities of the systems were determined. One unit of enzyme is defined as the amount which will cause a reduction of 20 per cent in the viscosity in sixty minutes. The reaction follows a unimolecular course and as a result one unit of enzyme causes a reduction of 10 per cent in 19 minutes, or 5 per cent in 8.2 minutes. Two units of the enzyme will cause a decrease of 20 per cent in viscosity in half the time required by one unit. An example of calculation will be given below.

*Amylase.*—For the determination of amylase, Davison's (1925) method was used. The limitations of the method have been discussed in an earlier paper (Chesley, 1931). The procedure was very similar to that used for the estimation of proteases. Sodium chloride was added to the system in a concentration of 0.05 M. The pH was kept at 7.2 by means of phosphate buffers. Units of enzyme are calculated in the same manner as for the proteases.

*Lipase.*—The Kanitz (1906) method was used. Ten milliliters of faintly alkalinized (NaOH) olive oil were incubated with 3 ml. of tissue extract for 16 hours at 40° C. To this were added, at the end of the digestion period, 50 ml. of acid-free alcohol and 5 ml. of ether, with three or four drops of phenolphthalein. The mixture was shaken and titrated with 0.1 N NaOH. A blank was run for each experiment. The amount of oleic acid liberated under the action of the enzyme was then calculated from the titration value.

## Experimental Results

### *Factors Influencing Enzyme Activity*

*Temperature.*—The results of these experiments will be the subject of a separate paper. It was found that over a period of thirty minutes, under the conditions of pH and ionic concentrations which were maintained, the optimal temperature is 35° C. Accordingly, this temperature was kept throughout the whole work.

*Hydrogen Ion Concentration.*—Experiments were done only with

amylase. The biliary amylase of several different fishes, acting at 35° C., was found to have a critical optimum at pH 7.2. The activities are considerably less at pH 7.0 and 7.4. This is shown in Table I; the values given are relative, the activity at pH 5.3 being set at unity. Sodium chloride was present in a concentration of 0.05 M, and phosphate buffers in an equal amount. These determinations were made viscometrically, and the optimum, which is atypically critical, may be governed in part by a pH effect upon the stability of the viscosity of the starch. Further investigation of pH effects upon fish amylase is planned.

As a routine procedure, the pH was maintained at 7.2 for all amylase determinations.

*Sodium Chloride Concentration.*—Since the chloride ion is the most efficacious of the anions activating amylase, and since the chloride concentration of the sea water is higher than the optimal activating concentration reported for mammalian amylase, the influence of chloride concentration upon fish amylase was studied, using three methods of measuring enzyme activity. The experiments were conducted as follows. Fifty-milliliter portions of 2 per cent soluble starch, buffered to pH 7.2 by means of 0.05 M phosphate mixture, were put into a series of flasks kept in the water bath at 35° C. Successively increasing quantities of a molar solution of NaCl were added to the preparations, and as soon as the temperature of the bath was attained, a definite quantity of enzyme solution was added. At intervals of a minute, five drops of the digest were removed and blown into 5 ml. of a N/8,000 iodine solution. The time was determined for the disappearance of the blue color reaction, and for the disappearance of any color reaction (referred to in Table II as "no blue" and "achromic" iodine methods respectively). At the end of 30 minutes, 5 ml. of the digest were removed and added to 95 ml. of Fehling's solution, and the maltose concentration was determined.

TABLE I

*The effect of pH upon fish amylase activity.* Temperature 35° C. Phosphate 0.05 M; NaCl 0.05 M. Time, 30 minutes. Determined viscometrically.

| Source | Relative activity | | | | | | |
|---|---|---|---|---|---|---|---|
| | pH : 5.3 | 5.9 | 6.6 | 7.0 | 7.2 | 7.4 | 7.7 |
| Rudderfish | 1 | 1.3 | 1.4 | 1.6 | 2.4 | 2.1 | 1.9 |
| Toadfish | 1 | 1.4 | 1.6 | 2.8 | 7.6 | — | 5.4 |
| Puffer | 1 | 1.7 | 2.2 | 2.7 | 3.4 | 3.2 | — |

The results, which are shown in Table II as relative activities at different chloride concentrations, indicate that the concentration of chloride which fully activates mammalian amylases (0.01 M, Sherman, Caldwell, and Adams, 1928) is not sufficient to activate fully fish amylase. In all routine determinations, the NaCl concentration was kept at 0.05 M, as this concentration fully activates the enzyme. Incidentally, these figures are not strictly quantitative, as the concentration of chloride impurities in the starch is unknown.

TABLE II

*The effect of chloride concentration upon fish amylase activity.* Temperature 35° C.; pH 7.2; phosphate 0.05 M. (Starch not chloride-free.)

| Method | Relative activity | | | | | |
|---|---|---|---|---|---|---|
| | None | 0.0025 M | 0.0125 M | 0.025 M | 0.05 M | 0.1 M NaCl |
| Iodine. No blue..... | 1 | 1.70 | 2.38 | 2.70 | 2.77 | 2.80 |
| Iodine. Achromic..... | 1 | 1.82 | 2.20 | 2.50 | 2.67 | 2.70 |
| Copper reduction..... | 1 | 1.56 | 1.96 | 2.16 | 2.56 | 2.60 |
| Mean..... | 1 | 1.69 | 2.18 | 2.45 | 2.67 | 2.70 |

*Concentration of Enzyme.*—As is to be expected, fish amylase obeys the general rule (which does not apply to certain proteases) that the rate of hydrolysis is proportional to the concentration of enzyme. Twice as much enzyme effects the same change in half the time. This was determined viscometrically over a range between 0.02 and 0.6 ml. of tissue extract, and is shown in Table III. The definition of the enzyme unit is based upon this relationship.

TABLE III

*The effect of enzyme concentration upon fish amylase activity.* Temperature 35° C.; pH 7.2; phosphate 0.05 M; NaCl 0.05 M. Determined viscometrically.

| Enzyme | Decrease in viscosity | Time required | Units per ml. enzyme sol. |
|---|---|---|---|
| *ml.* | *per cent* | *minutes* | |
| 0.02 | 5 | 70 | 5.85 |
| 0.04 | 5 | 35 | 5.85 |
| 0.08 | 5 | 15 | 6.80 |
| 0.16 | 10 | 20 | 6.00 |
| 0.32 | 10 | 10 | 6.00 |
| 0.64 | 20 | 15 | 6.24 |

## TABLE IV

*Proteases.* Units per gram of tissue. Temperature 35° C.; pH 2.3 and 7.4. Body weights 200 to 280 grams per fish.

| Fish | Stomach | Intestine | | | | Cæca | | Pancreas | | Liver | | Bile and gall bladder | | Total units in body (Approximate) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Upper third | | Second third | | Units | Wt. | Units | Wt. | Units | Wt. | Units | Wt. | |
| | | Units | Wt. | Units | Wt. | | | | | | | | | |
| Mackerel | — | 287 | 0.96 | — | — | 19 | 4.25 | 587 | 0.63 | 13 | 3.47 | — | — | 760 |
| Menhaden | Trace | 40 | 0.41 | Trace | 0.38 | 142 | 3.54 | 140 | 0.37 | Trace | 0.73 | 23 | 1.16 | 580 |
| Scup | 16 | 228 | 0.62 | Trace | — | 238 | 0.18 | 150 | 0.31 | 68 | 0.23 | 46 | 2.34 | 340 |
| Sea robin | 24 | 44 | 1.14 | — | 0.95 | 98 | 1.00 | 103 | 0.54 | Trace | 3.18 | 77 | | 250 |
| Toadfish | 22 | Trace | — | Trace | — | — | 0.00 | 2 | 0.45 | 28 | 7.60 | Trace | | 210 |

These figures will serve to illustrate the method of calculation of units of enzyme. One unit of the enzyme, by definition, will cause a 5 per cent decrease in viscosity in 8.2 minutes. Since 70 minutes were required for 0.02 ml. of enzyme solution to produce a 5 per cent change in viscosity, there are present $8.2/70 = 0.117$ unit, and in one ml. of the enzyme solution there would be $0.117 \times 1/0.02 = 5.85$ units.

### *Digestion in the Fishes Studied*

*The Mackerel.*—The mackerel is predominantly an animal plankton feeder, which means that it is an active fish. Its energy requirements would indicate that it should have a rapid and efficient digestion. That this is the case will be seen by reference to Tables IV, V, and VI, which show that the mackerel has the greatest amounts of enzymes. The pancreas, and secondarily, the intestine, are the chief sites of zymo-

TABLE V

*Amylases.* Units per gram. pH 7.2. Temperature 35° C. NaCl 0.05 M; phosphate 0.05 M.

| Fish | Stomach | Intestine | | Cæca | Pancreas | Liver | Bile | Total amylase |
|---|---|---|---|---|---|---|---|---|
| | | Upper third | Second third | | | | | |
| Mackerel | Neg. | Trace | 16 | Trace | 70 | Neg. | Neg. | 50 |
| Menhaden | Neg. | 12 | 24 | 21 | 60 | Trace | 6 | 40 |
| Scup | Neg. | Neg. | — | Neg. | 35 | Trace | 6 | 25 |
| Sea robin | Neg. | Neg. | Trace | 7 | Trace | Trace | 20 | 20 |
| Puffer | Neg. | 20 | — | — | 35 | 40 | 17 | — |
| Toadfish | Neg. | Trace | 3 | — | Trace | Trace | 5 | Trace |

poiesis. The pyloric cæca, of which there are about 190, produce very small quantities of enzymes except lipase. Amylase secretion is well localized in the pancreas.

*The Menhaden.*—The menhaden is another active fish, being a plankton feeder. As an adaptation to its plant diet, the menhaden intestine is much longer than that of any of the other fishes studied; also the stomach is remarkable for its heavy muscular walls and horny lining.

There is a large amount of body fat, to which the fish owes its economic importance. In view of their fatty metabolism, and the fats and oils in the diet, one might expect to find relatively much lipase in the digestive system. This is not the case.

In protease content, the menhaden is second only to the mackerel. The large number of cæca are important as a source of enzymes. The data are shown in Tables IV, V and VI.

*The Scup.*—A third fish of the active group was studied. Tables IV, V, and VI show that the scup has nearly as much protease, per gram of tissue, as the menhaden, while lipase and amylase are present in considerable amounts. The four pyloric cæca, small as they are, seem to be important in the secretion of protease. The bile, with which the intestinal content is invariably deeply stained, possesses a considerable amount of protease and some amylase. Apparently the bile is an important factor in the scup's digestion; the gall bladder is relatively large and always distended with enzyme-rich bile.

*The Sea Robin.*—This fish stores fat, and in the great majority of cases, the liver is cream-colored and greasy, indicating large amounts of liver fat. The gastro-intestinal lipase is slight in quantity, as in the menhaden; in fact it is the least found in any of the fishes studied

TABLE VI

*Lipases.* Grams of oleic acid per gram of tissue. Alkaline olive oil. Temperature 40° C.

| Fish | Stomach | Intestine | | Cæca | Pancreas | Liver | Bile |
|---|---|---|---|---|---|---|---|
| | | Upper third | Second third | | | | |
| Mackerel | 0.29 | 0.54 | — | 0.46 | 0.71 | 0.42 | 0.35* |
| Menhaden | — | 0.14 | 0.12 | 0.12 | 0.06 | 0.07 | 0.05 |
| Scup | 0.17 | 0.30 | — | 0.14 | 0.31 | 0.32 | — |
| Sea robin | Neg. | Trace | Trace | 0.02 | 0.10 | Trace | Trace |
| Puffer | — | 0.23 | — | — | 0.12 | 0.21 | Neg. |
| Toadfish | — | Neg. | Neg. | — | 0.03 | 0.12 | Trace |

* Gall bladder and bile. Only three values averaged; one of these was unusually high (0.78), possibly due to contamination by pancreatic tissue.

(Table VI). The trypsin and amylase are correlated in quantity with the general activity of the fish (Tables IV and V).

*The Puffer.*—Proteases were not estimated in the puffer. Tables V and VI show that the amylase and lipase are correlated with the general activity of the fish.

*The Toadfish.*—In this, the most sluggish of the species studied, the amounts of digestive enzymes, except lipase, are the least. The large liver is apparently the chief source of enzymes (Tables IV, V, and VI).

## DISCUSSION

Both Kenyon (1925) and Vonk (1928) have found that the herbivorous carp has much more amylase than have the carnivorous fishes. The present investigation shows that this does not apply in the case of

the menhaden, which feeds largely upon small plants. This diet is rich in oils, yet the menhaden appears to have but slight quantities of lipase.

Tables IV, V, and VI show clearly that the amounts of digestive enzymes, in the fishes studied, are a correlative of the general activity. At one extreme are found the mackerel and menhaden—fishes that probably never cease swimming and thus impose constant demands upon their energy stores. At the other extreme is the toadfish, with the other fishes ranging in between.

As for the distribution of enzyme production among the various organs concerned with digestion, no generalization can be made because of the limited number of species studied. However, in the mackerel, which has a compact pancreas, the production of enzymes is centered for the most part in that organ, while in the other species studied the pancreas is not well defined and enzyme secretion is not localized in it. In the menhaden, where the pancreas is somewhat disseminated through the cæca, both protease and amylase production reside here to a greater degree than in any other portion of the system, while lipase is secreted in several places. Lipase secretion is characteristic of the highly organized pancreas. In other fishes, in which the pancreas is even more diffuse, there are decreasing stages of localization of secretory function in that gland. Apparently a high degree of morphological organization is the concomitant of a high degree of physiological organization.

In the mackerel, which has a well-developed pancreas, the cæca play a relatively minor rôle in the secretion of enzymes. In the menhaden, scup, and sea robin, where the pancreas does not show a high degree of morphological and physiological differentiation, the cæca seem to be important in this respect. This is of particular interest since the cæca are embryologically equivalent to the pancreas.

From a consideration of the data in hand, it is difficult to decide if there is an accumulation of enzymes during fasting, but the general impression is that there is no piling up of enzymes in the secretory cells.

It is interesting to note that the biles are usually active enzymatically. In the case of the scup, the presence of enzymes in the bile is readily comprehensible, as sections of the liver show scattered acini of pancreatic tissue, and the liver extracts are active. However, in the sea robin the liver extracts are almost wholly inactive, the liver sections show no pancreatic tissue, and yet the bile contains a protease and amylase. Unfortunately, I have no sections of the sea robin gall bladder.

With the exception of the puffer, a rough correlation is shown between the amounts of amylase in the digestive systems, and the blood-sugar levels. Inasmuch as both of these parallel, in general, the activities of the fishes, there may be no deeper significant relation between the

two. Yet it seems probable that the efficacy of starch and glycogen digestion should condition the amount of carbohydrate taken into the body from the tract. Once in the system, part of the carbohydrate may go into storage as fat or glycogen, but part of it might well go toward maintaining the blood-sugar level. However, with the interconversion of foods and potential sources of glucose in the fat and protein, the fish could be more or less independent of its starch-digestive power.

## Conclusions

The biliary and pancreatic amylases of several marine fishes have a critical optimum at pH 7.2, when the temperature is 35° C., NaCl 0.05 M, phosphate buffer 0.05 M, and the period of digestion is 30 minutes.

The concentration of NaCl required to activate amylase fully is higher for fish amylase than for mammalian; for fish amylase, it was found that 0.05 M NaCl is sufficient.

The rate of digestion of starch varies inversely as the concentration of enzyme, over a considerable range.

The digestive enzymes parallel, in quantity, the general activity of the fishes studied. This is especially true of trypsin, but not of pepsin.

The menhaden, which subsists chiefly upon plant forms, is not adapted in having little lipase, since the diet is rich in oils.

Lipase is least in quantity in the fishes having a fatty metabolism (insofar as they store large reserves of fat). These are the menhaden and the sea robin.

In the fishes studied, the pancreas varies from a compact organ to an exceedingly diffuse structure. Apparently the degree of morphological organization is a concomitant of the degree of physiological organization. The compact pancreas is the center of enzyme secretion; the diffuse organ produces only a small portion of the enzymes poured into the enteron.

When the pancreas is not highly organized, the pyloric cæca seem partially to supplant it in enzyme secretion.

I wish to express my sincere gratitude to Professor F. G. Hall, under whose direction this work was done. I also acknowledge my indebtedness to the United States Bureau of Fisheries for the facilities afforded for the experiments.

## BIBLIOGRAPHY

Bigelow, H. B., and W. W. Welsh, 1924. Fishes of the Gulf of Maine. *Bull. U. S. Bur. Fish.*, **40**: 1.

Chesley, L. C., 1931. The Validity of the Viscometric and Wohlgemuth Methods for the Quantitative Determination of Amylase. *Jour. Biol. Chem.*, **92**: 171.

CHESLEY, L. C., 1934. The Influence of Temperature upon the Amylases of Cold- and Warm-blooded Animals. *Biol. Bull.*, in press.

DAVISON, W. C., 1925. A Viscometric Method for the Quantitative Determination of Amylase. *Johns Hopkins Hospital Bull.*, **37**: 281.

GRAY, I. E., AND F. G. HALL, 1930. Blood Sugar and Activity in Fishes with Notes on the Action of Insulin. *Biol. Bull.*, **58**: 217.

HALL, F. G., 1929. The Influence of Varying Oxygen Tensions upon the Rate of Oxygen Consumption in Marine Fishes. *Am. Jour. Physiol.*, **88**: 212.

HALL, F. G., AND I. E. GRAY, 1929. The Hemoglobin Concentration of the Blood of Marine Fishes. *Jour. Biol. Chem.*, **81**: 589.

KANITZ, A., 1906. Über Pankreassteapsin und über die Reaktiongeschwindigkeit der mittels Enzyme bewirkten Fettspaltung. *Zeitschr. physiol. Chem.*, **46**: 482.

KENYON, W. A., 1925. Digestive Enzymes in Poikilothermal Vertebrates. An investigation of enzymes in fishes, with comparative studies of those of amphibians, reptiles, and mammals. *Bull. U. S. Bur. Fish.*, **41**: 181.

NORTHROP, J. H., AND R. G. HUSSEY, 1923. A Method for the Quantitative Determination of Trypsin and Pepsin. *Jour. Gen. Physiol.*, **5**: 353.

PECK, J. I., 1894. On the Food of the Menhaden. *Bull. U. S. Bur. Fish.*, **13**: 113.

RIDDLE, O., 1909. The Rate of Digestion in Cold-blooded Vertebrates—the Influence of Season and Temperature. *Am. Jour. Physiol.*, **24**: 447.

SHERMAN, H. C., M. L. CALDWELL, AND M. ADAMS, 1928. Influence of Concentration of Neutral Salt on the Activation of Pancreatic Amylase. *Jour. Am. Chem. Soc.*, **50**: 2535.

SULLIVAN, M. X., 1907. The Physiology of the Digestive Tract of Elasmobranchs. *Bull. U. S. Bur. Fish.*, **27**: 3.

SVOLIMA, A. F., 1919. The Digestion of Fishes. *Russ. Physiol. Jour.*, **2**: 170. (*n. v.*)

VAN SLYKE, D. D., AND G. F. WHITE, 1911. Digestion of Protein in the Stomach and Intestine of the Dog-fish. *Jour. Biol. Chem.*, **9**: 209.

VONK, H. J., 1928. Over einige enzymen in het darmkanal van lagere dieren. *Nederl. tijdschr. voor geneesk.*, **72**: 466.

YUNG, E., 1899. Recherches sur la digestion des poissons. *Arch. Zool. Expér.*, Series 3, **7**: 121. (Extensive bibliography and survey of the field.)

# EGG-TRINUCLEARITY IN HABROBRACON[1]

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## Mosaics from Binucleate Eggs

In the parasitic wasp *Habrobracon juglandis* (Ashmead) specimens are occasionally found which are mosaic for various hereditary traits. The theory has been advanced (Whiting, P. W., 1924) that these mosaics develop from binucleate eggs in which two nuclei (oötids from one oöcyte) of different genetic constitution take part in cleavage. Data have recently been summarized with respect to various genes (Whiting, P. W., 1932*b*).

Three groups of mosaics may be distinguished with respect to sex—females, gynandromorphs, and males. Thus far only one mosaic female from a binucleate egg has been reported (Greb, 1933, p. 183). The mother was heterozygous for the factor aciform, *ac*, which affects the antennæ and the genitalia. The father was aciform. The mosaic was aciform on one side, wild-type on the other. It is supposed that both egg nuclei, one containing aciform, the other its normal allelomorph, were fertilized by sperm with the recessive factor aciform.

Many gynandromorphs have been reported, for the most part coming from binucleate eggs with one nucleus fertilized. Thus the male parts are haploid and matroclinous, the female parts diploid and biparental.

Mosaic males develop from unfertilized binucleate eggs laid by heterozygous mothers. Such males have previously been reported mosaic for a large number of different characters. Males mosaic for various other traits have been found recently among the offspring of females heterozygous for these traits. The new factors involved in male mosaicism are crescent, *cr* (eyes); droopy, *dr* (wings); gynoid, *gy* (antennæ and abdominal sclerites); honey, *ho* (body color); carrot, *wh*$^{c}$ (eyes). The eye color differences in the mosaics involving the

[1] The material here reported has been found by students and independent investigators at the University of Pittsburgh and at Pennsylvania College for Women, Pittsburgh, Pa. Study of specimens and preparation of the manuscript was done for the most part at Woods Hole and Cold Spring Harbor. Technical assistance has been furnished by the Committee on Effects of Radiation on Living Organisms (National Research Council).

locus white were clear-cut without any modification or intergradation, both in the case of carrot vs. black (wild-type) and of carrot vs. white. The latter are produced from carrot-white compound females.

Males in *Habrobracon* usually develop from unfertilized eggs, but when the mother is mated to a closely related male some of the sons are biparental. This is shown by the fact that they inherit paternal traits as well as maternal. They are diploid in chromosome number and sterile or produce very few offspring. It was therefore to be expected that rarely a mosaic male would be produced instead of a gynandromorph from a fertilized binucleate egg. The fertilized nucleus might develop into diploid male parts instead of female while the unfertilized nucleus would develop into haploid male parts as in the gynandromorph. Such a haplo-diploid male mosaic was reported (Whiting, P. W., 1932*b*, pp. 305–306) resulting from a cross of an orange-eyed female by a closely related black-eyed male. The eyes showed both black and orange regions and the specimen produced many orange daughters which were highly fertile. A second haplo-diploid male mosaic (No. 675) has recently been found by R. L. Anderson. One eye was orange, one black. When this male was crossed with an orange-eyed female, many orange-eyed daughters were produced and also three black-eyed daughters. The orange daughters were of normal fertility, but the three black proved sterile although they lived for several days and fed on caterpillars. Thus the gonads as well as the eyes were mosaic with haploid (orange) and diploid (black) regions and the male bred in part as a normal haploid, in part as a nearly sterile biparental male.

Several males mosaic for honey body color were also mosaic for other factors affecting the same structures as are affected by honey. Thus No. 627 had left wings honey, right shot-veins; No. 660 had left legs honey, right stumpy; No. 662 had left wings wild-type, right honey shot-veins and left legs stumpy, right honey; No. 645 had left antenna long, right honey, and the wings all long.

Several males have been found, mosaic for various factors of Chromosome II. The genes located in this group with map distances are: crepe-wings, *cw*,—13—honey, *ho* (body-color)—22—cantaloup, *c* (eyes),—11—long, *l* (antennae, wings, legs),—2—narrow, *n* (wings), —25—defective, *d* (R4 wing-veins). No. 643 (found by Forrest W. Miller) was from a mother heterozygous (*c.l.d/cw.ho*) for five of these factors. Left antenna was honey, right wild-type; eyes were mosaic for cantaloup; wings were crepe-wings; some legs were honey and others wild-type. The mosaic was bred to wild-type females and 20 daughters were tested by breeding. Results showed that 16 of the sperm of the

mosaic contained *cw.c.d* while 4 contained *cw.ho.d*. According to the theory of origin of the two genetically different cleavage nuclei from the same oöcyte, the division separating the two chromatids which remained in the egg from the two that passed into the first polar nucleus was reductional for *cw, l* and *d* but equational for *ho* and *c*. A triple crossover may have occurred between two strands, a double between the other two.

Egg binuclearity will explain the origin of the mosaics above cited since not more than two combinations of the various factors are evident in their bodies or by breeding test.

## Mosaic Males from Trinucleate Eggs

The origin of several males (found by Raymond J. Greb) produced by virgin females heterozygous for honey and for stumpy cannot be explained by egg binuclearity since in each case the legs represent three of the four possible combinations of the maternal factors. As an example, legs of No. 659 (Figs. 1–6) are wild-type, stumpy and honey stumpy but none are stumpy alone. Other arrangements are represented in different specimens but none involving more than three of the four combinations of factors.

The first example of a mosaic male from a trinucleate egg was found by Kathryn A. Gilmore. A heterozygous female (*c.l/n.d*) produced a mosaic male (No. 511) (Fig. 9) with narrow wings on the left side while the right was long (antennæ, wings, legs), defective (R4 veins), cantaloup (eyes). Legs of the right side were long. The factor long shortens the wings especially toward the tip, which in the primary is curved ventrally. It also lengthens the segments of the antennæ and the legs. Four matings were made between mosaic male No. 511 and cantaloup females. The first produced cantaloup females only; the second (two days later) produced wild-type only; the third (four days later) and the fourth (two days later) cantaloup females only. Fourteen of the cantaloup daughters showed by breeding test that they carried long and defective, while 33 of the wild-type daughters carried no recessives except cantaloup from their mothers. The mosaic therefore bred in part as cantaloup, long, defective; in part as wild-type. Trinuclearity is involved in the genetic composition of this male. The right side of the body and the gonads in part represent a single crossover (between *n* and *d*) strand. The left wings represent the alternative strand. The gonads in part represent a single crossover (between *l* and *n*) strand.

## Genetic Stratification of Gonads

A point of interest to be noted in the breeding behavior of the mosaic male (No. 511) described above is genetic stratification of the gonads. Sperm were in the first mating, *c.l.d;* in the second wild-type; while in the third and fourth they were again *c.l.d.*

Previous breeding tests of males with mosaic gonads have shown that there may be a slight tendency toward stratification (Whiting, P. W., and Anna R. Whiting, 1927, p. 98) or the two kinds of sperm may be produced in approximately equal numbers throughout the life of the male (Whiting, P. W., 1928, p. 299).

Genetic stratification in the ovaries was suggested in connection with the mutation to vestigial (Whiting, P. W., 1932*a*, p. 24) in which case 50 non-vestigial were produced during the early life of the mother (in vials *a, b, c*); 12 non-vestigial and 12 vestigial subsequently (in vial *d*); and 7 non-vestigial finally (in vial *e*).

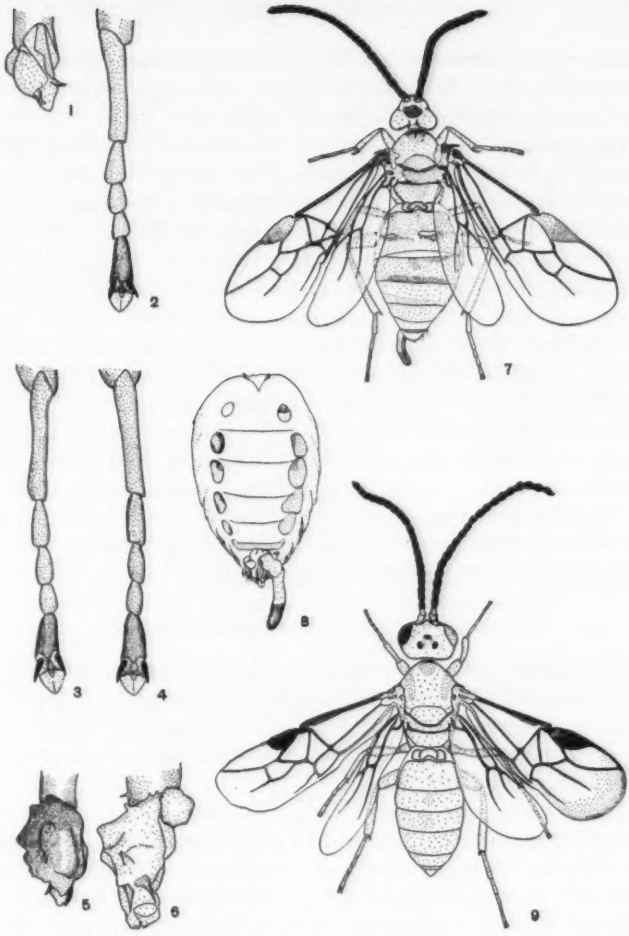
One ovary of the female producing the mutants attenuated (*Jour. Gen.*, in press) may have been stratified. In vials *a–c* there were 32/46 (69.56 per cent) attenuated, while in vials *d–j* there were 46/140 (32.86 per cent) attenuated. The difference is statistically significant, suggesting that posterior regions of both ovaries were heterozygous but that in anterior regions only one ovary contained the mutant factor.

In the case of a recent mutation, leglike antennæ (found by Raymond J. Greb), no leglike among 40 males occurred in vials *a–b*; 2 leglike among 19 males occurred in vial *c*; while 14 leglike among 22 males occurred in vials *d–e*.

## A Trinucleate Gynandromorph

Gynoid, a factor for weak male intersexuality, increases sclerotization of the abdomen and shortens the antennæ of males, thus making them resemble females superficially. Gynoid females are indistinguishable in appearance from wild-type females.

In connection with studies on the nature of biparental males, Magnhild Torvik-Greb crossed gynoid females with eyeless males. Offspring were gynoid males and wild-type (biparental) males and females as expected. Gynoid is closely linked with eyeless (map distance 7). One fraternity contained in addition to these three expected classes a gynandromorph (No. 640) (Fig. 7) displaying three genetically distinct types of tissue. The head was typical eyeless with left antenna normal male. The right antenna was defective toward the tip showing considerable fusion of segments. The abdomen (Fig. 8) was female on the left side, gynoid male on the right except that the first right




EXPLANATION OF PLATE I

FIGS. 1–6. Tarsi of male No. 659, mosaic for honey and for stumpy. × 80.

FIG. 1. Left prothoracic, honey stumpy.

FIG. 2. Right prothoracic, wild-type.

FIG. 3. Left mesothoracic, wild-type.

FIG. 4. Right mesothoracic, wild-type.

FIG. 5. Left metathoracic, stumpy.

FIG. 6. Right metathoracic, honey stumpy.

FIG. 7.* Dorsal view of gynandromorph No. 640, mosaic for eyeless and for gynoid. × 14.

FIG. 8. Ventral view of abdomen of gynandromorph No. 640. Note female, gynoid male, and wild-type male sternites. × 22.

FIG. 9. Dorsal view of male No. 511, mosaic for long (antennæ and wings), for narrow (wings) and for cantaloup (eyes). × 13.

sternite was characteristic for wild-type male, probably genetically eyeless like the head. Genitalia consisted of a complete male set and a large sensory gonapophysis (female) on the left.

The specimen may have started development as a female from a normally fertilized egg. Some sort of somatic segregation may then have given rise to the two types of male tissue, eyeless and gynoid.

An alternative hypothesis is that of egg binuclearity with fertilization of one nucleus to form the female parts and cleavage of a supernumerary sperm nucleus as well as of the unfertilized oötid to form the two types of male tissue. Development from a sperm nucleus (androgenesis or merogony) must be very rare if it occurs at all in *Habrobracon*. A gynandromorph (No. 523) produced by a black-eyed female (stock No. 25) crossed with an orange-eyed male (stock No. 3) had a male head with orange eyes. The recessive paternal trait appearing in the male parts may be explained by androgenesis or by somatic segregation. Among offspring from similar crosses made later with the same stocks there occurred a gynandromorph with male parts matroclinous (black eyes) as expected from the general rule. The question of androgenesis therefore remains open.

### Impaternate Gynandromorphs

With only two exceptions all the gynandromorphs found in *Habrobracon*, amounting to over 150, have occurred in bisexual fraternities among the progeny of mated females. Whenever the female parts could show a paternal effect this was apparent, indicating that gynandromorphs come from fertilized eggs.

The two exceptional impaternate gynandromorphs (found by Kathryn A. Gilmore) occurred in the course of experiments in production of impaternate females. Females of a certain strain of Minnesota stock crossed with various males give rise to females which if bred as virgins occasionally produce a daughter. This is contrary to the usual rule of parthenogenetic production of males only, arrenotoky. Genetic analysis indicates that such daughters develop as a result of suppression of the second oöcyte division, in other words from a diploid nucleus. Impaternate gynandromorphs would be expected to occur in this material if in addition to a second oöcyte nucleus a reduced nucleus, an oötid from division of the first polar nucleus, took part in cleavage. An impaternate gynandromorph would then be comparable to a trinucleate haploid male mosaic except that one diploid nucleus failed to undergo reduction.

The first impaternate gynandromorph (No. 522) was found August,

1931, among the offspring from a virgin female heterozygous for tapering (antennæ) and for white (eyes), *ta/wh*. Left antenna was female (14 segments), right was male (21 segments). Eyes and ocelli were wild-type (black), the latter female (small). The abdomen was entirely female. Thorax was asymmetrical, being female (lighter in color) on the left side, male on the right. Since neither male nor female antenna nor male nor female regions of the eyes showed the mutant traits for which the mother was heterozygous, it may be supposed that the female parts were heterozygous for both genes, while the male parts carried both dominants.

The second impaternate gynandromorph was produced by a virgin female heterozygous for crescent (eyes). The head was male (23 segments in each antenna) with large black ocelli and normal eyes; the abdomen was female. Male parts were thus non-crescent but the character of female parts is indeterminable.

## LITERATURE CITED

GREB, RAYMOND J., 1933. Effects of Temperature on Production of Mosaics in Habrobracon. *Biol. Bull.*, **65**: 179.

WHITING, P. W., 1924. Some Anomalies in Habrobracon and their Bearing on Maturation, Fertilization and Cleavage. *Anat. Rec.*, **29**: 146.

WHITING, P. W., 1928. Mosaicism and Mutation in Habrobracon. *Biol. Bull.*, **54**: 289.

WHITING, P. W., 1932*a*. Mutants in Habrobracon. *Genetics*, **17** (No. 1): 1.

WHITING, P. W., 1932*b*. Modification of Traits in Mosaics from Binucleate Eggs of Habrobracon. *Biol. Bull.*, **63**: 296.

WHITING, P. W., AND ANNA R. WHITING, 1927. Gynandromorphs and other Irregular Types in Habrobracon. *Biol. Bull.*, **52**: 89.

WHITING, P. W., AND ANNA R. WHITING, 1934. A Unique Fraternity in Habrobracon. *Jour. Genetics* (in press).

# A NEW TYPE OF SEX-INTERGRADE[1]

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Two types of sex-intergrades have been clearly distinguished (Goldschmidt, 1931) having certain regions of the body apparently male, other regions female. In gynandromorphs male and female regions differ from each other not only phenotypically but in genetic constitution as well. Intersexes, on the other hand, have all parts of the body of similar genetic make-up. Male and female regions appear phenotypically different but their chromosomal constitution is identical. Intersexes are classed as either male or female. Male intersexes begin their development as males but organs developed later are feminized. Female intersexes begin their development as females but organs developed later are masculinized.

A third type of sex-intergrade is reported in the present paper. These are genetically male mosaics with feminized genitalia. No genotypically female tissue is present, but the two types of male tissue interact to produce the feminization. Mosaic males with feminized genitalia resemble gynandromorphs and have for convenience been called gynandroid.

In the parasitic wasp, *Habrobracon juglandis* (Ashmead), normal (zygotic, diploid) females develop from fertilized eggs, normal (azygotic, haploid) males from unfertilized eggs. Diploid males of biparental inheritance (showing dominant traits of both parents) occur in small numbers from certain types of crosses. Such males have diploid sperm and are sterile or nearly sterile. When they have dominant factors and are crossed with recessive females, their few offspring are triploid females showing the dominant traits of their diploid father. There is no evidence of intersexuality in diploid males or in triploid females unless the reduced size of ovaries and near sterility of the latter indicate this. Moreover, their reproductive reactions are entirely normal, the diploid males mating readily and the triploid females stinging caterpillars and feeding and ovipositing upon them.

[1] The material here reported has been found by students and independent investigators at the University of Pittsburgh and at Pennsylvania College for Women, Pittsburgh, Pa. Study of specimens and preparation of the manuscript was done for the most part at Woods Hole and Cold Spring Harbor. Technical assistance has been furnished by the Committee on Effects of Radiation on Living Organisms (National Research Council).

### Gynandromorphism

Gynandromorphs occasionally appear in bisexual fraternities. Gynandromorphic offspring from a recessive female by a dominant male show the maternal trait in the male (haploid) parts, the paternal trait in the female (diploid) parts. This evidence is consistent with the theory of egg binuclearity. Fertilization of one nucleus results in the female parts; development of the other without fertilization results in the male parts. Gynandromorphs may have male heads, female abdomens, or the reverse; one side may be male, the other female; anterior left and posterior right may be of one sex, the remainder of the other sex; male islands may occur in female regions (Fig. 4) or female islands in male regions (Figs. 1 and 25). When genitalia are mixed in sex, there may be a full set of male structures and a half set of female structures. Two of these cases have been published (Whiting, P. W., and Anna R. Whiting, 1927. Page 114 and Plate I, Figs. 17–21) (Whiting, P. W., and Edward J. Wenstrup, 1932. Page 33 and Figs. 13*c* and 14). A third specimen of this type (No. 509) is female on the left side of the body (short antenna, larger wings, larger abdominal sternites), male on the right. The dorsal side of the abdomen (Fig. 2) is asymmetrical, showing male coloration on right side, female on left. The half set of female structures is placed laterally and anterior to the full male set (Fig. 3), a fact which may furnish cause for speculation as to homologies of parts.

### Weak Male Intersexuality

A single mutant-type male found in a bisexual fraternity by Raymond J. Greb illustrates weak male intersexuality. Antennæ are short as in the female (Fig. 10) and abdominal tergites (Fig. 8) and sternites (Fig. 9), especially in the anterior part of the abdomen, are heavier than in the normal male, thus approaching the condition found in the female. Ocelli are large, of normal male size; genitalia are of normal male character. Instincts are typical for the male and mating readily takes place. The mutant-type called gynoid, *gy*, is of normal viability, segregating in approximately equal numbers among offspring from heterozygous mothers. Females heterozygous for gynoid have thus far produced five males mosaic for gynoid. Gynoid females have been derived by mating heterozygous females to gynoid males. They are structurally indistinguishable from wild-type females, are of normal viability and fertility and produce, as virgins, gynoid males only. A gynoid stock has been derived which breeds true.

Gynoid has been tested for linkage with many other genes. It proved to be closely linked with eyeless, *el*. Counts from heterozygous

virgin females (*el/gy*) gave wild-type 112, eyeless 1157, gynoid 1484, eyeless gynoid 80; 6.7 per cent crossovers. When gynoid is crossed with non-gynoid, the biparental males are wild-type as is the case with any other recessive trait. Reciprocal crosses between gynoid and eyeless have been made by Magnhild M. Torvik. Ten eyeless females crossed with gynoid males produced wild-type females 296, wild-type (biparental) males 43, eyeless males 155. Sixty-five matings of the biparental males resulted in only nine females to 3372 males. These females were sterile. Twenty-three gynoid females (10 orange and 13 heterozygous for orange) crossed with eyeless males produced wild-type females 794, wild-type (biparental) males 30, gynoid males 435. None of the daughters or biparental sons were orange, while approximately 50 per cent of the gynoid sons from heterozygous mothers (+/o) and all of the gynoid sons from orange mothers were orange as expected. Twenty-four matings of the biparental sons resulted in no daughters and 1566 males.

Heredity of this factor for weak male intersexuality is therefore in every way similar to that of other recessives.

## Feminized Genitalia in Mosaic Males

Virgin mothers heterozygous for various traits occasionally produce a son mosaic for one or more of these traits. These mosaic males develop from unfertilized eggs in which polar nuclei as well as the reduced egg nucleus presumably take part in parthenogenetic cleavage.

Normal male genitalia (Fig. 27) consist of a median penis on either side of which is an inner clasper made up of a basal part and a movable terminal part. Laterad to these is the pair of outer claspers surmounted by several bristles. The claspers are connected proximally with a tri-

### Explanation of Plate I

Fig. 1. Ventral view of abdomen of gynandromorph (No. 482), showing male genitalia and sternites with a female island indicated by a large sternite. × 22.

Fig. 2. Dorsal view of abdomen of gynandromorph (No. 509), showing pigmental asymmetry, female on left, male on right. × 22.

Fig. 3. Ventral view of genitalia of gynandromorph (No. 509) with complete male set posteriorly and female gonapophyses (sensory and two elements of sting) on left. × 80.

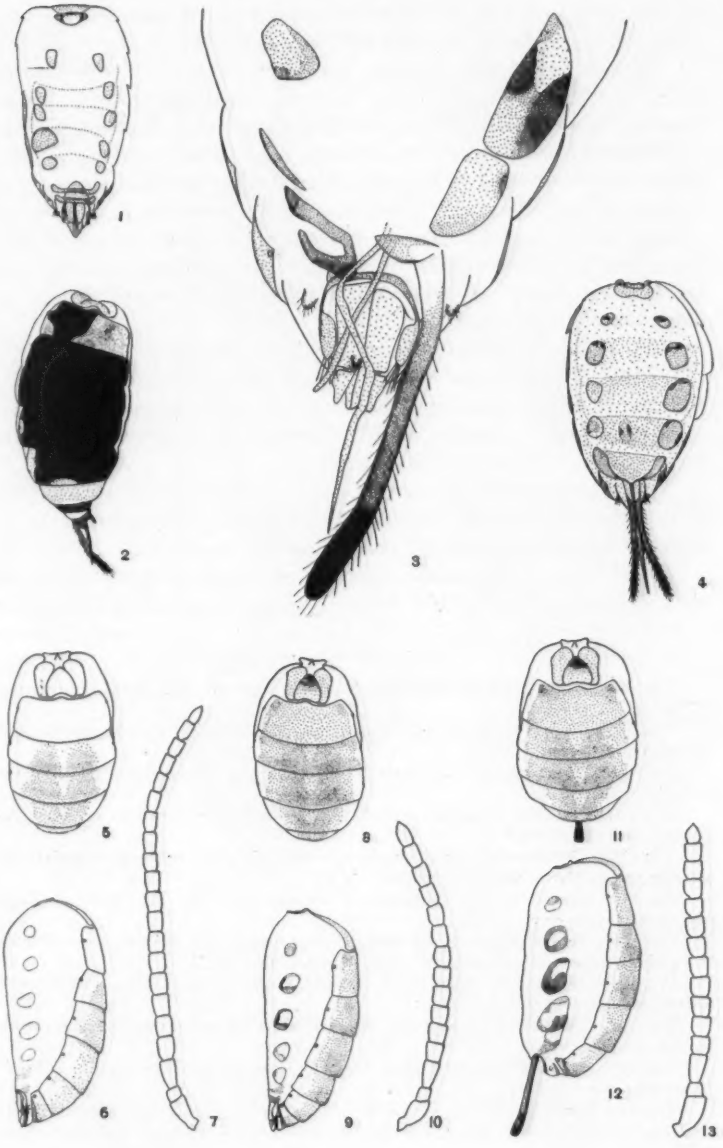
Fig. 4. Ventral view of abdomen of gynandromorph (No. 499), showing female genitalia and sternites with a male island indicated by two small sternites. × 22.

Figs. 5, 8, 11. Antero-dorsal views of abdomens of wild-type male, gynoid male, and female respectively. × 22.

Figs. 6, 9, 12. Lateral views of abdomens of wild-type male, gynoid male, and female respectively. × 22.

Figs. 7, 10, 13. Antennæ of wild-type male, gynoid male, and female respectively. × 33.

PLATE I



angular basal piece which may be withdrawn into what appears ventrally as the sixth and seventh abdominal segments. A pair of cerci surmounted by bristles are seen laterad to the genitalia.

Marked genital abnormalities are characteristic of certain mutant types, but occur rarely in other cultures. Among 299 mosaic males, however, there were 36 showing striking abnormality, including extreme displacement, reduplication or deficiency of parts, and feminization. Males showing the last trait have been called gynandroid because they suggest gynandromorphs. Genitalia of large numbers of non-mosaic siblings of mosaic males have been carefully examined and counts from miscellaneous cultures have often involved examination of genitalia more or less closely. Genital abnormalities in these are much less frequent than in their mosaic sibs.

No. 666 was a gynoid male mosaic for cantaloup eye color. Genitalia were almost normal (Fig. 29), but the right outer clasper was feminized laterally showing a small protuberance suggesting a female structure, a sensory gonapophysis. Posterior sternites were heavier on the right side, indicating feminization.

No. 678 was a gynoid male mosaic for cantaloup. Genitalia (Fig. 28) consist of a complete male set plus a sensory gonapophysis on the right (which conceals the right outer clasper in the drawing).

No. 652 was gynoid on the right side, wild-type on the left. Dorsal view of abdomen (Fig. 14) shows correspondingly heavier anterior

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EXPLANATION OF PLATE II

FIG. 14. Dorsal view of abdomen of mosaic male No. 652, showing pigmental asymmetry. × 22.

FIG. 15. Ventral view of mosaic male No. 652, showing gynoid antennæ and sternites on right. × 22.

FIG. 16. Genitalia of mosaic male No. 652, showing feminization and bilateral reduplication. × 80.

FIG. 17. Genitalia of mosaic male No. 579, showing feminization on right, deficiency on left. × 80.

FIG. 18. Genitalia of mosaic male No. 653, showing extreme feminization; sensory gonapophysis and sting on left. × 80.

FIG. 19. Dorsal view of abdomen of mosaic male No. 601, showing slight pigmental asymmetry. × 22.

FIG. 20. Ventral view of abdomen of mosaic male No. 601, showing darkening and thickening of sternites on right, feminization. × 22.

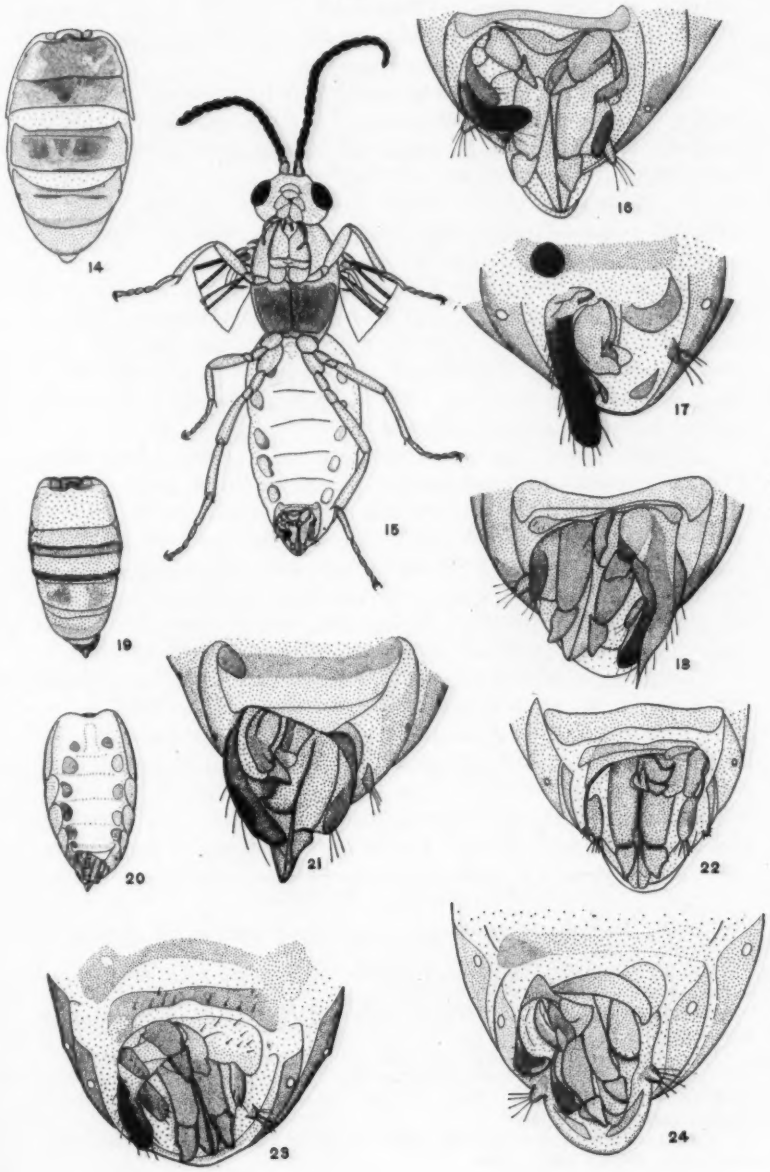
FIG. 21. Genitalia of mosaic male No. 601, showing feminization and reduplication on right. × 80.

FIG. 22. Genitalia of mosaic male No. 497, showing reduplication but no feminization. × 80.

FIG. 23. Genitalia of mosaic male No. 603, showing feminization and reduplication on right. × 80.

FIG. 24. Genitalia of mosaic male No. 659, showing reduplication but no feminization. Asymmetry in pigment is due to mosaicism for honey, *ho*, body color. × 80.

PLATE II



tergites dextrally. Ventral view (Fig. 15) shows right abdominal sternites and right antenna characteristic for gynoid. Genitalia (Fig. 16) had sensory gonapophysis on right and complete reduplicated male set of genitalia cephalad to the terminal set.

No. 579 was mosaic for ivory eye color. Genitalia (Fig. 17) were lacking on the left. A well-developed sensory gonapophysis with a median lobe occurred on the right and also an inner clasper showing reduplication. Outer claspers were lacking.

No. 653 was mosaic for ivory, shot-veins and stumpy. Genitalia (Fig. 18) consisted of a complete male set with reduplicated male parts, a sting and a sensory gonapophysis on the left.

No. 601, mosaic for white eyes, showed pigmental asymmetry on dorsal side of abdomen (Fig. 19), as is frequent in mosaics. Enlargement of sternites is seen ventrally (Fig. 20) on the feminized right side. Genitalia (Fig. 21) showed sensory gonapophysis and reduplicated male parts on the right. The right inner clasper in the normal position lacked the terminal segment.

No. 603, mosaic for yellow antennæ and reduced wings, shows (Fig. 23) sensory gonapophysis and reduplicated male parts on right.

Reduplicated male parts occurring in mosaics which show no feminization are illustrated by No. 497 (Fig. 22) and No. 659 (Fig. 24). The latter male was mosaic for honey body color and it may be clearly seen that the line dividing honey from non-honey regions passes through the genitalia. The difference is especially well-marked in the outer claspers, left being honey and right (reduplicated) being non-honey.

Asymmetry in number of antennal segments has been shown (Whiting, P. W., 1932) to be correlated with male mosaicism. More or less asymmetry in growth of genitalia has been noted in several mosaic males in which there is neither reduplication nor deficiency of parts. Thus either the inner or the outer claspers may be of different size or may originate more anteriorly on one side than on the other. The entire anal region as indicated by the cerci may be shifted laterally so that one cer-

EXPLANATION OF PLATE III

FIG. 25. Ventral view of abdomen of gynandromorph No. 596, showing female island. × 22.

FIG. 26. Genitalia of gynandromorph No. 596, showing either female island or feminization of male genitalia. × 80.

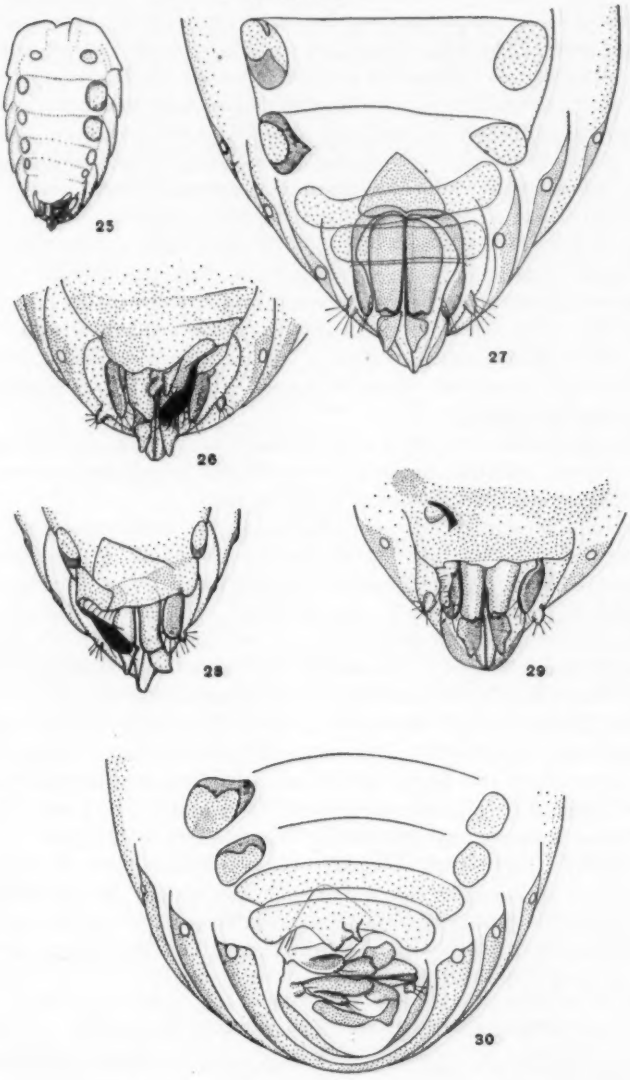
FIG. 27. Normal genitalia of mosaic male No. 647, showing gynoid abdominal sternites on right. × 80.

FIG. 28. Genitalia of mosaic male No. 678, showing feminization. × 80.

FIG. 29. Genitalia of mosaic male No. 666. The right outer clasper is definitely but slightly feminized. There is slight pigmental asymmetry of abdominal sternites posteriorly. × 80.

FIG. 30. Genitalia of mosaic male No. 664, placed transversely as if developed from gynoid (right) side. × 80.

PLATE III



cus is moved cephalad, the other caudad. A striking example of asymmetry in genitalia is illustrated by No. 664, a mosaic for gynoid and for cantaloup. The genitalia (Fig. 30) originate from the right side and extend toward the left. Right side is gynoid (note heavier sclerites, both sternites and tergites), left is non-gynoid. Difference in growth rate of the genetically different tissues may explain the anomaly. The sclerotized rudiments ventrad and cephalad to the transverse genitalia may represent deficient left claspers and half of penis. Structures of the right may therefore be reduplicated by a mirror image.

Genitalia of a mosaic male were figured (Whiting, P. W., 1928, Plate I, Fig. 2), showing a reduplicated right outer clasper and other structures suggesting an extra right inner clasper and penis. (This male was sterile, probably on account of inability to copulate.)

A male (No. 685) not obviously mosaic had a complete set of normal male genitalia and in addition a well-developed feminized gonapophysis to the left of the left outer clasper. This male proved sterile in four observed matings.

Another male (No. 693) not obviously mosaic had, in addition to the complete male set, an extra inner clasper toward the base at the left. There was no evidence of feminization.

Thirty-six cases of striking abnormality occurred among the 299 mosaic males. Three showed decided malformation or displacement but no feminization, reduplication, or deficiency. The remaining 33 included 22 feminized and 11 not feminized. Among the feminized 3 showed reduplication of male parts as well as deficiency, 13 showed reduplication only, and 3 showed deficiency only. Of those not feminized 8 showed reduplication only and 3 showed deficiency only.

The 23 males with feminized genitalia (including besides the 22 mosaics, No. 685 which was not obviously mosaic) were summarized with reference to the side on which deficiency or reduplication of male parts appeared. The summary (Table I) indicates that there is high correlation between feminization and reduplication with respect to the side affected. Of the 22 feminized on one side only, 15 showed reduplication on the same side only, while one showed reduplication on both sides. There appears to be no correlation between feminization and deficiency, for only one showed deficiency on the feminized side alone while five showed it on both.

### Genital Abnormality and Genital Mosaicism

Since feminization and other abnormalities of genitalia are relatively frequent in mosaics, the question presents itself as to whether this condition may be due to the presence of genetically distinct tissues in the

genital region. The majority of the mosaics bred lacked definite factor differences visibly affecting the abdomen. The following cases, however, may be cited involving the factors miniature (body size), honey (body color), and gynoid (which enlarges and thickens abdominal sclerites).

TABLE I

*Classification of mosaic males with feminized genitalia according to side of body showing reduplication or deficiency in male genitalia.*

| | Side of body showing | |
|---|---|---|
| Feminization | Reduplication | Deficiency |
| Right....14 | Neither....4 | Neither....2<br>Both....2 |
| | Right....9 | Neither....7<br>Both....2 |
| | Both....1 | Neither....1 |
| Left....8 | Neither....2 | Neither....1<br>Both....1 |
| | Left....6 | Neither....5<br>Left....1 |
| Both....1 | Neither....1 | Neither....1 |

No. 343 was entirely miniature on the left side, non-miniature on the right. The difference apparently extended through the genitalia, which were markedly deficient, consisting of a left outer clasper and penis only.

No. 664 was gynoid on right, non-gynoid on left in the region of the genitalia, which were transversely placed (Fig. 30).

No. 659 showed much reduplication. The genitalia were divided into honey and non-honey regions (Fig. 24).

No. 645 showed genitalia with right side honey, left non-honey and containing many reduplicated parts.

Nos. 660, 661, 662 each had a complete male set honey, but small sensory gonapophyses non-honey; No. 661 had reduplicated male parts honey while No. 662 had reduplicated male parts non-honey.

These cases show that abnormality may be associated with genetic diversity. However, No. 647 (Fig. 27) indicates genetic diversity of tissues but normal genitalia and No. 648 having the right outer clasper honey, left non-honey, and genitalia normal, proves that genitalia may be mixed without necessarily causing abnormality.

### Feminization of Abdominal Sternites

Mosaicism for honey body color may cause asymmetry of sternal pigmentation but not of sternal sclerotization, which is much greater in the female than in the male. Eighteen of the 23 males showing feminization of genitalia had abdominal sternites approximately symmetrical in size while five had marked asymmetry. In two of these this asymmetry was due to mosaicism for gynoid, the gynoid tissue being on the side showing the feminized gonapophysis in one case (No. 652, Fig. 15) but on the opposite side in the other (No. 665).

Attention has already been called to asymmetry in sternites of No. 601 (Fig. 20), which was non-gynoid, and to No. 666 (Fig. 29), which was completely gynoid. In the case of No. 656 also, the last two sternites were larger on the left side, which showed both feminization and reduplicated male elements. These three cases of sternal asymmetry not due to mosaicism for gynoid and with heavier sternites on the side showing feminized genitalia suggest that the cause for feminization of genitalia and of sternites may be the same.

### Gynandromorphs with Mixed Genitalia

Gynandromorphs with mixed genitalia have been found. Three (Nos. 5, 446, 509) had well-developed sensory gonapophyses and one or two elements of the sting on one side with a complete male set of genitalia terminally (Fig. 3).

A fourth gynandromorph (No. 673) was female on the left side of the body, male on the right. There were no external male genitalia, but on the left side were a long sensory gonapophysis and two elements of the sting, one of which was very long and thin.

A fifth gynandromorph (No. 596) was in general female on the left side, male on the right, showing much asymmetry of color. This asymmetry extended into the abdomen both dorsally and ventrally, the fifth tergite showing the sex difference. Ventrally the asymmetry extended through the third sternite, but posterior to that sternites were male (Fig. 25). Genitalia (Fig. 26) were male except for a small sensory gonapophysis and some minute rudiments of the sting or reduplicated parts.

A sixth gynandromorph (No. 689), which was anteriorly female, showed asymmetry in wings and in abdominal sclerites, the left side being female, the right male. Genitalia were very deficient, showing a minute sensory gonapophysis on the left and a small median structure which was possibly a penis.

The possibility should be mentioned that the last two gynandromorphs may have been also gynandroid with feminized male rather than

female elements in the genitalia. In this connection should be recalled the male genitalia of a fertile gynandromorph previously reported, No. 395 (Whiting, P. W., and E. J. Wenstrup, 1932, p. 36), with a reduplicated outer clasper on the right, female, side.

### Feminized Genitalia in Habrobracon brevicornis (Wes.)

"Intersexual males" of *Habrobracon brevicornis* (Wes.), a species parasitic on the European corn-borer, *Pyrausta nubilalis* Hübn., have been reported (Whiting, P. W., and Anna R. Whiting, 1927. Pages 112, 113, 115, Plate II) having in addition to the normal male set of genitalia a small "female" gonapophysis. One (No. 275) with very irregular asymmetrical abdominal sternites and short antennæ occurred in a bisexual fraternity containing also a gynandromorph and three males (?) with short antennæ and asymmetrical sclerites. Since this fraternity contained females, it is possible that the "intersexual males" may have been gynandromorphs.

A second, No. 193, occurring in a male fraternity and hence probably from an unfertilized egg, had antennæ of normal male length, abdominal sternites typical for the male, but with a small "female" gonapophysis located, as in No. 275, on the right side at the base of the normal male set of genitalia.

A previously unreported male of *H. brevicornis* had, in addition to a normal male set of genitalia at the tip of the abdomen, a second complete set on the left side near the base of the typical set. The left external clasper of this extra set was somewhat thickened and of slightly darker color than normal while its right external clasper was black and small with cross lines suggesting a female sensory gonapophysis.

Thus it is suggested that a condition similar to gynandroid may occur in *H. brevicornis*. No factor differences indicating mosaicism were available, however.

### Discussion

The theory of sex-determination in Hymenoptera recently advanced (Whiting, P. W., 1933. *Biol. Bull.*, **65**: 357) was first suggested by feminization of genitalia in mosaic males. It was postulated that the mosaic is composed of two types of tissue each recessive for a different sex factor, the dominant allelomorphs to both of which are necessary for femaleness. One type of tissue, $X$, contains sex factors $F$ and $g$, while the other, $Y$, contains their allelomorphs $f$ and $G$. Females $X + Y$ are diheterozygous, $F.g/f.G$, and normally produce two types of males, $X$ and $Y$, genetically different but phenotypically similar.

The analogy was drawn with mosaicism for eye color. A male may

have eyes mosaic for two recessive whites (white, *wh*, and ivory, $o^i$) genetically different but phenotypically similar. The dominant allelomorph to ivory present in the white region apparently causes the production of some substance that diffuses through and interacts with the dominant allelomorph to white, producing black pigment at the border in the ivory region. No diffusion seems to take place in the reverse direction. Similarly in feminized mosaics factor *G* in the *Y* region of the body causes some substance that diffuses through and interacts with factor *F* in the *X* region, producing female-like structures at the border in the *X* region.

It is to be noted that reduplication of male genitalia is relatively frequent in mosaics and is highly correlated with the side showing feminization. It may likewise be recalled that in the less pronounced cases it is the outer clasper that shows feminization. These facts suggest that the gonapophysis is a reduplicated and modified outer clasper and that reduplication is one phase of feminization. Thus it may be that the eight mosaics showing reduplication but no feminization are like those showing feminization in that they are composed of *X* and *Y* tissue with the reduplicated structures on the *X* side.

Not all genital irregularities in mosaic males, however, are to be ascribed to interaction of *X* and *Y*. Deficiency of parts has no correlation with the side feminized and many types of distortion may be due merely to genetically diverse growth rates, as in the case of antennal asymmetry.

Normality of genitalia in mosaics is to be ascribed in the majority of cases to non-mosaicism of the genital region or, in those cases in which the genitalia are mosaic, to the fact that both regions are *X* or both *Y*. Mosaicism would then not involve the sex factors but other differences.

The rare cases of reduplication or feminization occurring in males not obviously mosaic may be ascribed to origin from binucleate eggs in which no visible genetic difference was involved or to failure of bodily structures to exhibit the genetic mosaicism.

Mixed genitalia in gynandromorphs are usually attributable to the presence of both female ($X + Y$) and male ($X$ or $Y$) tissue. It is, however, possible that some cases arise from trinucleate eggs in which one nucleus was fertilized while the other two ($X$ and $Y$) gave rise to genetically diverse male tissue and gynandroid genitalia.

Feminization of abdominal sternites is less frequent than that of genitalia but the three cases cited were on the same side of the body as the feminized genitalia. Asymmetry of ocelli has been observed in some mosaic males but this trait is too variable for definite conclusions. The

markedly increased antennal asymmetry of mosaic males cannot be ascribed to a feminizing influence. No shortening of antennæ suggesting feminization has been found in mosaic males, nor is there any feminization of instincts. Preliminary survey of gonads and of other internal structures has as yet yielded no evidence of feminization.

## LITERATURE CITED

GOLDSCHMIDT, RICHARD, 1931. Die Sexuellen Zwischenstufen. Monographien aus dem Gesamtgebiet der Physiologie der Pflanzen und der Tiere, 23: 437–455. Julius Springer, Berlin.

WHITING, P. W., 1928. Mosaicism and Mutation in Habrobracon. *Biol. Bull.*, **54**: 289.

WHITING, P. W., 1932. Asymmetry of Wild-Type Traits and of Secondary Sexual Characters in Mosaics of Habrobracon. *Jour. Exper. Zoöl.*, **62**: 259.

WHITING, P. W., 1933. Sex-Determination in Hymenoptera. *Biol. Bull.*, **65**: 357.

WHITING, P. W., AND EDWARD J. WENSTRUP, 1932. Fertile Gynandromorphs in Habrobracon. *Jour. Hered.*, **23**: 31.

WHITING, P. W., AND ANNA R. WHITING, 1927. Gynandromorphs and other Irregular Types in Habrobracon. *Biol. Bull.*, **52**: 89.

# BIPARENTAL MALES AND BIPARENTAL RATIOS IN HABROBRACON

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Genetic evidence for the diploid nature of biparental males in the parasitic wasp, *Habrobracon juglandis* (Ashmead), has been reported by Anna R. Whiting (1927) and Magnhild M. Torvik (1931). It was demonstrated that biparental males were diploid for the seventeen mutant factors tested, and no evidence was found to indicate that they were simplex for a sex chromosome, as had been suggested. Magnhild M. Torvik showed cytologically that biparental males have twice as many spermatogonial chromosomes as occur in azygotic males. P. W. Whiting and R. L. Anderson (1932) reported evidence inconsistent with the theory that the loss of a sex chromosome in gametogenesis causes fertilized eggs to develop into males rather than females. The percentage (or ratio) of males among biparentals (biparental males $\times$ 100/biparentals) was shown to be increased with increase of temperature or degree of relationship and to be negatively correlated with proportion of biparentals (biparentals $\times$ 100/total). They concluded that some condition arising at fertilization or subsequent to it determines the sex of biparentals.

In the investigations reported at this time these two percentages were studied in four experiments. In one a successful attempt was made to increase the proportion of biparental males by selection. In the second experiment orange eye-color from stock No. 3 was graded up to wild-type stock No. 11, giving females having various proportions of the original material from Nos. 3 and 11. A similar experiment was carried out for another orange-eyed stock, *D-o*, and wild-type stock No. 25, and a fourth which involved *D-o* and wild-type stock No. 31.

The origin of the stocks used will be described in the account of the separate experiments. In the making of crosses matings were observed and each male was left with the female from four to six hours. The females were then placed singly in shell vials and fed four or five host caterpillars, and the vial termed *a*. Four days later each female was transferred to a new vial, marked *b*, and fed again. Transfers were made in turn every four days until the females had been in vials *e* for

four days. At that time the females were discarded, since they tend to exhaust their supply of sperm shortly after that time and thenceforth give rise to azygotic males only. The cultures were kept in incubators regulated for a constant temperature of 30° C., except for short intervals during which transfers were made from one vial to another.

The author is indebted to Professor P. W. Whiting, at whose suggestion the investigation was begun, for guidance during the progress of the work, and to Professor Anna R. Whiting for advice and criticism. Acknowledgment is also made to the Committee on Effects of Radiation on Living Organisms of the National Research Council for technical assistance through grants to Professor P. W. Whiting.

## The Influence of Selection

An experiment designed to show the effect of selection on the percentage of males among biparentals and on the percentage of fraternities including such males was carried through eleven generations by D. R. Charles, and from that point through the thirty-first generation by the author.

The experiment was begun by crossing orange-eyed females (stock 19*c*) to black-eyed males (wild-type stock No. 1). The offspring included orange-eyed azygotic males (*o*), a few black-eyed biparental males (*O*), and black-eyed females (*Oo*). Reference to Table I will show that five of the sixty fraternities contained biparental males. The heterozygous females, *Oo*, which were sisters to biparental males, were allowed to mate with their azygotic brothers, *o*. The $F_2$ generation included then *Oo* and *oo* females, and *O* and *o* males. Biparental males could not be detected in this generation. To produce the $F_3$, *oo* females were mated to their *O* brothers. Five of the 34 fraternities contained biparental males. The $F_4$ was produced in the same way as the $F_2$, and the $F_5$ as the $F_3$. The strain was continued in this way, always by brother-sister matings, through the $F_{21}$ generation. It will be seen that the odd-numbered generations contained biparental males which could be distinguished from azygotic males, and that the even generations were used in continuing the strain.

Considering all the fraternities in each of the odd generations (Table I), there is to be seen a steady rise in the percentage of males among biparentals up to the ninth generation, from which time the value is more or less constant, except for small fluctuations. The drop from 10 per cent of the $F_9$ to 6.5 of the $F_{11}$ (difference, $3.5 \pm 1.2$ [1]), may have been due to chance selection or to some unknown environmental factor. The

[1] Standard errors are used throughout this paper.

TABLE I

*Selection Experiment*

| Generation | Number of matings | Percentage with +♂♂ | ○♂♂ | +♂♂ | +♀♀ | Biparental males × 100 / Biparentals | Biparentals × 100 / Total |
|---|---|---|---|---|---|---|---|
| $F_1$ | 5 | | 32 | 7 | 32 | 17.9±6.1 | 53.9±5.9 |
| | 55 | | 477 | 0 | 547 | | 53.4±1.6 |
| Total | 60 | 8.3 | 509 | 7 | 579 | 1.2± .4 | 53.5±1.5 |
| $F_3$ | 5 | | 44 | 6 | 20 | 23.1±8.3 | 37.1±5.8 |
| | 29 | | 175 | 0 | 151 | | 46.3±2.8 |
| Total | 34 | 14.7 | 219 | 6 | 171 | 3.4±1.3 | 44.7±2.5 |
| $F_5$ | 20 | | 162 | 36 | 246 | 12.8±2.0 | 63.5±2.3 |
| | 16 | | 93 | 0 | 123 | | 56.9±3.8 |
| Total | 36 | 55.6 | 255 | 36 | 369 | 8.9±1.4 | 61.4±1.9 |
| $F_7$ | 48 | | 532 | 77 | 606 | 11.3±1.2 | 56.2±1.4 |
| | 31 | | 307 | 0 | 282 | | 47.8±2.1 |
| Total | 79 | 60.8 | 839 | 77 | 888 | 8.0± .9 | 53.5±1.1 |
| $F_9$ | 63 | | 1022 | 152 | 1109 | 12.1± .9 | 55.2±1.0 |
| | 24 | | 236 | 0 | 259 | | 53.2±2.2 |
| Total | 87 | 72.4 | 1258 | 152 | 1368 | 10.0± .8 | 54.7± .9 |
| $F_{11}$ | 126 | | 3372 | 356 | 4400 | 7.5± .4 | 58.5± .6 |
| | 32 | | 708 | 0 | 739 | | 51.1±1.3 |
| Total | 158 | 79.7 | 4080 | 356 | 5139 | 6.5± .3 | 57.3± .5 |
| $F_{13}$ | 36 | | 1082 | 145 | 1130 | 11.4± .9 | 53.7±1.0 |
| | 4 | | 132 | 0 | 84 | | 38.8±3.3 |
| Total | 40 | 92.5 | 1214 | 145 | 1214 | 10.7± .8 | 52.4±1.0 |
| $F_{15}$ | 12 | | 298 | 25 | 391 | 6.0±1.1 | 58.2±2.2 |
| | 5 | | 136 | 0 | 141 | | 50.9±3.0 |
| Total | 17 | 71.6 | 434 | 25 | 532 | 4.5± .9 | 56.2±1.8 |
| $F_{17}$ | 10 | 100. | 106 | 22 | 226 | 8.9±1.8 | 70.1±2.4 |
| $F_{19}$ | 16 | | 625 | 55 | 529 | 9.4±1.2 | 48.3±1.4 |
| | 1 | | 97 | 0 | 32 | | 24.8±3.7 |
| Total | 17 | 94.1 | 722 | 55 | 561 | 8.9±1.1 | 46.0±1.3 |
| $F_{21}$ | 15 | | 415 | 59 | 434 | 11.9±1.4 | 54.3±1.6 |
| | 3 | | 61 | 0 | 84 | | 57.9±4.0 |
| Total | 18 | 83.3 | 476 | 59 | 518 | 10.2±1.2 | 55.0±1.5 |

$F_{15}$ generation was bred when an incubator was not available and the low percentage may have been due to the variable temperature or to chance selection. Percentages for the seventeenth, nineteenth, and twenty-first generations are about the same, indicating that no further increase by selection was being obtained.

If one considers only those fraternities including biparental males, the percentage of males among biparentals shows no significant rise or deviation from one generation to another. Obviously then the increase in the percentage for all fraternities is due to an increase in the percentage of matings producing biparental males. These percentages show a steady rise up through the $F_{13}$ generation, after which they fluctuate around an average of 85 or 90.

Since the ratios of males among biparentals were practically the same for the seventeenth, nineteenth, and twenty-first generations, selection seemed no longer effective. To determine whether or not the strain had become genetically homogeneous through selection and close inbreeding (brother-sister matings), parents for generations 23 through 31 were selected on a different basis than through 21. Through the $F_{21}$ generation orange females and black males, which were crossed to produce biparental males, were always offspring of black, heterozygous females which were sisters to biparental males. From the $F_{21}$ generation the parents of the odd generations were chosen from fraternities, of the preceding odd generation, having no biparental males or having a percentage termed high, medium, or low. Percentages from 0–7 were termed low, from 7–14, medium, and from 14 up, high.

In the $F_{23}$ generation there were three groups of fraternities (Table II, column 2, "basis of selection") whose parents were derived from fraternities having, respectively, average percentages of 17.1, 9.5, and zero. All three groups had, however, approximately the same ratio. The same results were obtained in the $F_{25}$ generation but in the $F_{27}$ chance selection was apparently effective in an opposite direction. Parents from several fraternities having an average percentage of 9.8, medium, gave offspring showing a percentage of $3.8 \pm 1.07$. Parents from fraternities having a low percentage, averaging 6.3, gave offspring showing a value of $19.1 \pm 2.25$. The difference between the two is very significant from the statistical standpoint, being $15.3 \pm 2.49$. The cause of this difference may have been due, in addition to the factor of chance, to the occurrence of one or more mutations affecting the production of biparental males.

Table I shows the ratios of biparentals for fraternities including biparental males and for those without them. No significant difference exists between the two groups of fraternities in generations 1, 3, 5, 9,

15, or 21, while in the other generations the fraternities including biparental males have significantly higher ratios of biparentals. In one of these, however, only a single fraternity lacked biparental males, and the low biparental ratio of a single fraternity may be due to fertilization by a male below the average in fertility. The differences are really significant in generations 7, 11, and 13. The meaning of this will be discussed later when comparisons are made with other experiments.

TABLE II

*Selection Experiment: Results of Selection in Several Directions*

| Generation | Basis of selection | Frats. with biparental* ♂♂ / Total frats. | ○ ♂♂ | + ♂♂ | + ♀♀ | Biparental males × 100 / Biparentals | Biparentals × 100 / Total |
|---|---|---|---|---|---|---|---|
| | 17.1 | 12/12 | 367 | 46 | 379 | 10.8 | 53.6 |
| $F_{23}$ | 9.5 | 11/12 | 424 | 44 | 386 | 10.2 | 50.3 |
| | none | 10/10 | 288 | 30 | 325 | 8.5 | 55.2 |
| Total | | 33/34 | 1079 | 120 | 1090 | 9.8 | 53.3 |
| | 16.2 | 9/12 | 354 | 21 | 347 | 5.7 | 50.8 |
| $F_{25}$ | 12.0 | 12/13 | 438 | 25 | 446 | 5.3 | 50.7 |
| | 5.1 | 12/15 | 563 | 24 | 448 | 5.1 | 45.6 |
| Total | | 33/40 | 1345 | 70 | 1241 | 5.3 | 49.3 |
| | 9.8 | 7/13 | 403 | 12 | 302 | 3.8 | 43.8 |
| $F_{27}$ | 6.3 | 6/6 | 128 | 30 | 127 | 19.1 | 55.1 |
| Total | | 13/19 | 531 | 42 | 429 | 8.9 | 47.0 |
| | 20.0 | 2/2 | 23 | 4 | 38 | 9.5 | 64.6 |
| $F_{29}$ | none | 6/7 | 66 | 16 | 86 | 15.6 | 60.9 |
| Total | | 8/9 | 89 | 20 | 124 | 13.8 | 62.2 |
| | 20.0 | 3/3 | 107 | 6 | 116 | 4.9 | 53.3 |
| $F_{31}$ | 9.4 | 10/10 | 357 | 36 | 396 | 8.3 | 54.7 |
| | none | 4/4 | 141 | 8 | 201 | 3.8 | 59.5 |
| Total | | 17/17 | 605 | 50 | 713 | 6.5 | 55.8 |

* Parents were selected from several fraternities whose parents belonged to fraternities with the average ratio of biparental males indicated.

## The Effect of Grading

### *The 11-o Experiment*

Matings of orange-eyed females of stock No. 3 to black-eyed males of wild-type stock No. 11 do not produce biparental males, since the stocks are not related. The *Oo* daughters of such matings were crossed to their *o* sons or nephews, and *oo* females obtained. These *oo* females may be thought of as being composed of one-half stock No. 3 material and one-half stock No. 11 material. When 44 of these were mated to males of stock No. 11, biparental males were present among the offspring. Orange was continually graded up to stock No. 11. Females (*oo*), having less and less of the genetic material of stock No. 3, except the eye-color, were crossed to males of stock No. 11. The females used in the eighth series of matings were 3/512 stock No. 3 and 509/512 stock No. 11.

Considering first all fraternities for each series (Table III), one can see a gradual rise in the percentage of males among biparentals. The highest point was reached in the fifth series, the percentage being 10.6 $\pm$ 1.8 greater than in the sixth. In the seventh and eighth the percentage went up again, being 21.6 in the eighth. The drop from the fifth to the sixth was probably due to unconscious selection of hereditary factors.

If one considers the fraternities in each series which included biparental males, it is seen that the percentage begins as 9.1 and jumps to 17.0 in the second series. There is a gradual rise until the fifth, when the highest point, 29.0, is reached. Then follows a gradual drop until the value is 21.6 in the eighth.

Grading of stock No. 3 up to stock No. 11 was successful not only in increasing the proportion of fraternities containing biparental males but also in raising the percentage among those fraternities. Comparison of the two groups of fraternities also leads one to believe that two different kinds of hereditary factors regulating the production of biparental males are present. One kind allows such males to be produced, and another kind modifies their ratio. From the fifth to the sixth series there was a considerable drop in the percentage for all fraternities, but a much smaller drop among those fraternities containing biparental males. This suggests that there was unintentional selection against the factors which cause the production of these males, but no selection against the modifying factors.

Comparison of biparental ratios for fraternities with and without biparental males shows the ratio to be less in every series for those fraternities having biparental males. The difference is highly signifi-

TABLE III

*11–o Experiment*

| Composition of ♂♂ ♀♀ | Number of matings | Percentage with +♂♂ | o ♂♂ | +♂♂ | +♀♀ | Biparental males × 100 / Biparentals | Biparentals × 100 / Total |
|---|---|---|---|---|---|---|---|
| 1/2 No. 3 | 17 | | 252 | 28 | 280 | 9.1±1.6 | 55.0±2.1 |
| | 27 | | 277 | 0 | 645 | | 70.1±1.5 |
| Total | 44 | 38.6 | 529 | 28 | 925 | 2.9± .5 | 60.2±1.2 |
| 1/4 No. 3 | 11 | | 167 | 39 | 190 | 17.0±2.5 | 57.9±2.5 |
| | 18 | | 185 | 0 | 448 | | 70.8±1.8 |
| Total | 29 | 37.9 | 352 | 39 | 638 | 5.8± .9 | 65.8±1.5 |
| 1/8 No. 3 | 17 | | 541 | 254 | 725 | 25.9±1.4 | 64.4±1.2 |
| | 11 | | 174 | 0 | 605 | | 77.6±1.5 |
| Total | 28 | 60.7 | 715 | 254 | 1330 | 16.0± .9 | 68.8±1.0 |
| 1/16 No. 3 | 7 | | 173 | 85 | 226 | 27.3±2.5 | 64.3±2.5 |
| | 2 | | 71 | 0 | 179 | | 71.6±2.8 |
| Total | 9 | 77.7 | 244 | 85 | 405 | 17.3±1.7 | 66.6±1.7 |
| 1/32 No. 3 | 22 | | 639 | 362 | 885 | 29.0±1.3 | 66.1±1.1 |
| | 2 | | 31 | 0 | 120 | | 79.5±2.3 |
| Total | 24 | 91.6 | 670 | 362 | 1005 | 26.5±1.2 | 67.1±1.0 |
| 1/64 No. 3 | 11 | | 306 | 101 | 281 | 26.4±2.2 | 55.2±1.9 |
| | 6 | | 107 | 0 | 251 | | 70.1±2.4 |
| Total | 17 | 65.7 | 413 | 101 | 532 | 15.9±1.4 | 60.5±1.5 |
| 1/128 No. 3 | 16 | | 321 | 107 | 402 | 21.0±1.8 | 61.3±1.7 |
| | 2 | | 14 | 0 | 52 | | 78.8±5.0 |
| Total | 18 | 88.8 | 335 | 107 | 454 | 19.1±1.7 | 62.6±1.6 |
| 3/512 No. 3 | 25 | 100.0 | 1156 | 369 | 1339 | 21.6±1.0 | 59.6± .9 |

cant, except in one series, the fourth, where the difference is over twice the standard error. This negative correlation suggests that fewer biparentals are produced when biparental males occur.

*The 25-o Experiment*

An experiment was begun by mating 12 females of *D-o* stock to males of wild-type stock No. 25, collected originally in New York City and inbred for a number of years. *D-o* is an orange-eyed stock derived

from the selection experiment described above. The 12 matings produced 411 orange males and 1067 black females. As expected, no biparental males were obtained, since the two stocks were unrelated. Some of the daughters, *Oo*, were mated to their *o* sons or nephews, and produced *Oo* and *oo* daughters. Twenty-three of the *oo* females, in composition one-half *D-o* and one-half stock No. 25, were crossed to males of 25, and biparental males were present among the offspring. By continual breeding up to 25, *oo* females were obtained which were in turn 1/4, 1/8, 1/16, and 1/32 *D-o*. Females of each group were crossed to males of stock No. 25 (Table IV).

TABLE IV

*25-o Experiment*

| Composition of *oo* ♀ ♀ | Number of matings | Percentage with +♂♂ | *o* ♂♂ | +♂♂ | +♀♀ | Biparental males × 100 / Biparentals | Biparentals × 100 / Total |
|---|---|---|---|---|---|---|---|
| 1/2 *D-o* | 7 | | 263 | 55 | 396 | 12.2±1.5 | 63.2±1.8 |
| | 16 | | 516 | 0 | 1291 | | 71.4±1.1 |
| Total | 23 | 30.4 | 779 | 55 | 1687 | 3.2± .4 | 70.9± .9 |
| 1/4 *D-o* | 5 | | 151 | 11 | 172 | 6.0±1.7 | 54.8±2.7 |
| | 11 | | 274 | 0 | 679 | | 71.2±1.5 |
| Total | 16 | 31.2 | 425 | 11 | 851 | 1.3± .4 | 67.0±1.3 |
| 1/8 *D-o* | 11 | | 149 | 22 | 290 | 7.0±1.4 | 67.6±2.2 |
| | 13 | | 222 | 0 | 528 | | 70.4±1 6 |
| Total | 24 | 45.9 | 371 | 22 | 818 | 2.6± .5 | 69.4±1.3 |
| 1/16 *D-o* | 15 | | 596 | 121 | 868 | 12.2±1.0 | 62.4±1.2 |
| | 9 | | 359 | 0 | 789 | | 68.8±1.4 |
| Total | 24 | 62.6 | 955 | 121 | 1657 | 6.8± .6 | 65.1± .9 |
| 1/32 *D-o* | 20 | | 712 | 104 | 790 | 11.6±1.1 | 55.7±1.2 |
| | 4 | | 141 | 0 | 368 | | 73.2±2.0 |
| Total | 24 | 83.2 | 853 | 104 | 1158 | 8.2± .7 | 59.7±1.1 |

Considering all fraternities, the percentage of males among biparentals was 3.2 in the first series, followed by a small drop to the second, and thereafter a gradual increase, until in the fifth series the percentage was 8.2. The very small difference between that and the

preceding suggested that further grading could not materially have increased the production of biparental males.

Consideration of fraternities containing biparental males shows a similarity to the 11-*o* experiment. In the initial crosses the percentage was $12.2 \pm 1.5$ as compared to $3.2 \pm .42$ for all fraternities. In the second and third series the value fell to 6.0 and 7.0, respectively, and rose again to 12.2 and 11.6 in the fourth and fifth. Since the per-

TABLE V

*31-o Experiment*

| Composition of *oo* ♀ ♀ | Number of matings | Percentage with +♂♂ | o ♂♂ | +♂♂ | +♀♀ | Biparental males × 100 / Biparentals | Biparentals × 100 / Total |
|---|---|---|---|---|---|---|---|
| 1/2 *D-o* | 9 | | 264 | 53 | 375 | 12.4±1.6 | 61.8±1.8 |
| | 19 | | 434 | 0 | 1236 | | 74.0±1.1 |
| Total | 28 | 32.1 | 698 | 53 | 1611 | 3.2± .4 | 70.4± .9 |
| 1/4 *D-o* | 15 | | 569 | 156 | 463 | 25.2±1.7 | 52.1±1.4 |
| | 1 | | 17 | 0 | 37 | | 68.5±6.3 |
| Total | 16 | 93.7 | 586 | 156 | 500 | 23.8±1.6 | 52.9±1.4 |
| 1/8 *D-o* | 16 | | 350 | 90 | 390 | 18.7±1.8 | 57.8±1.7 |
| | 4 | | 115 | 0 | 184 | | 61.5±2.8 |
| Total | 20 | 80.0 | 465 | 90 | 574 | 13.5±1.3 | 58.3±1.4 |
| 1/16 *D-o* | 4 | | 112 | 72 | 155 | 31.7±3.1 | 66.9±2.5 |
| | 6 | | 53 | 0 | 169 | | 76.1±2.8 |
| Total | 10 | 40.0 | 165 | 72 | 324 | 18.2±1.9 | 70.6±1.9 |
| 1/32 *D-o* | 17 | | 593 | 141 | 463 | 23.3±1.7 | 50.5±1.4 |
| | 6 | | 318 | 0 | 485 | | 60.4±1.7 |
| Total | 23 | 73.8 | 911 | 141 | 948 | 12.9±1.0 | 54.4±1.1 |

centage was no greater in the fifth than in the first, it suggests that in this experiment factors were present allowing the reaction to take place necessary for the production of biparental males, but that modifying factors were not present. In the 11-*o* experiment both kinds of factors were present, while the modifying factors were not present or of little importance in the selection experiment.

As in the 11-*o* experiment, the percentage of biparentals is less in

every case for fraternities including biparental males. The difference is highly significant except in one series, where it is 2.8 ± 2.7.

### *The 31-o Experiment*

An experiment was begun by crossing 10 females of *D-o* stock to males of wild-type stock No. 31, taken in Pittsburgh, Pennsylvania, in 1929 and inbred since that time. The 10 matings produced 316 orange males and 724 black females. In the same manner as for the 25-*o* experiment, this line was bred up to males of 31, *oo* females being in turn 1/2, 1/4, 1/8, 1/16, and 1/32 *D-o*. Females of each group were crossed to males of stock No. 31 (Table V).

The results of the 31-*o* experiment are more similar to those of the 11-*o* experiment than to those of the 25-*o* experiment. As regards all fraternities, the percentage of males among biparentals happened to be the same in the first cross as it was in the 25-*o* experiment. There is, however, a very significant rise from 3.2 to 23.8 in the second series of crosses. In the third series the value was significantly less, in the fourth it went up again, and in the fifth down. These fluctuations must have been due to chance selection of factors regulating the number of such males produced. Study of the fraternities containing biparental males suggests that such modifying factors are present, as in the 11-*o* experiment. Differences between the 25-*o* and 31-*o* experiments are probably due to the differences in residual heredity of the two type stocks, since females of the same stock were used to initiate the two experiments.

It is interesting to note that for the selection experiment, from which the *D-o* stock was derived, the highest percentage of males among biparentals was 13.8 ($F_{29}$). The highest percentage for the 25-*o* experiment was 8.2 and for the 31-*o* experiment 23.8. This indicates further a difference between stocks Nos. 25 and 31 in regard to factors regulating male biparentalism.

As in the 11-*o* and 25-*o* experiments, the percentage of biparentals is less in every generation for those fraternities including biparental males. The difference is significant in every case except one, in which it is 3.7 ± 3.3.

## The Influence of Age of Mother

In 1930 D. R. Charles reported that the percentages of males among biparentals decrease with increasing age of mothers. Percentages were calculated for the culture vials through which each female was successively passed, and were as follows: (*a*) 11.96 ± .34; (*b*) 7.71 ± .57;

(*c*) 7.77 ± .73; and (*d*) 5.41 ± .75. The two significant drops, from *a* to *b*, and from *a* to *d*, are 4.25 ± .67 and 6.55 ± .82, respectively.

Charles summarized data for the odd generations of the selection experiment through the eleventh generation. A summary by vials for generations 13, 19, 21, 23, 25, 27, and 31 is included in Table VI. Numbers for separate generations were too small to be significant, but the summary for all generations combined shows a decrease from vials *a* to *d*, and a very small rise in *e*. The significant drops are from *a* to *c*, 5.0 ± 1.1; from *a* to *d*, 6.2 ± 1.1; from *a* to *e*, 5.4 ± 1.5; and from *b* to *d*, 3.4 ± 1.1.

The 11-*o*, 25-*o*, and 31-*o* experiments were summarized in the same way, the data being recorded in Table VI. Separate generations indicated no decrease in the percentage with age of mother, and all generations combined, where the numbers are large enough to be significant, showed no difference from one vial to another. Actually, in the 11-*o* and 25-*o* experiments, vials *d* have the largest ratio, but the value does not differ significantly from any other.

TABLE VI

*Percentages of Males among Biparentals for Successive Periods in the Life of Females*

| Experiments | Vials | | | | |
|---|---|---|---|---|---|
| | *a* | *b* | *c* | *d* | *e* |
| Selection | 12.6± .79 | 9.8± .78 | 7.6± .7 | 6.4± .8 | 7.2±1.3 |
| 11-*o* | 24.1±1.2 | 22.6±1.1 | 22.9±1.3 | 26.1±1.3 | 23.8±1.6 |
| 25-*o* | 14.7±1.4 | 9.8±1.1 | 10.4±1.2 | 10.6±1.3 | 9.5±1.4 |
| 31-*o* | 21.5±1.7 | 21.5±1.6 | 21.4±1.7 | 25.1±2.3 | 18.1±2.6 |

No explanation will be offered at this time for the fact that in one experiment the percentage of males among biparentals decreases with increasing age of mothers, and in three other experiments percentages do not differ from one set of vials to another.

The 11-*o* experiment was also summarized to show the variations in the percentage of biparentals among total offspring with increasing age of mothers. Since the proportion of biparentals depends partly on the number of sperms received at mating, it was to be expected that this ratio would decrease with increasing age of the mothers or the passing of time from mating. The combined data for all generations showed a significant drop from *a* to *b*, *b* to *c*, and *c* to *e*, but not from *c* to *d*. Examination of the data for the separate series of matings showed that

when in *a* vials the percentage of biparentals is higher than the average in *a* vials for all the series in the experiment, a higher percentage is maintained in each of the five vials through which the females are transferred. When the percentage for *a* vials for one series is less than the average, a lower percentage is found throughout all the vials.

## Correlation of the Ratios

Attention has been called to the higher percentage of biparentals shown by groups of fraternities having no biparental males as contrasted with groups of fraternities having such males. Since a lower ratio of biparentals was thus found to be associated with the presence of biparental males, it was suspected that comparisons of the ratios within fraternities containing biparental males might show negative correlation. The correlation value was found to be $.157 \pm .075$ for 170 fraternities in the selection experiment, and to be $.189 \pm .081$ for the 141 fraternities of the 11-*o* experiment. Thus in these two experiments a low positive correlation was found to exist between the ratios.

Magnhild M. Torvik, summarizing data collected from experiments conducted by E. J. Wenstrup, found a low negative correlation to exist between the ratios, the value being $-.192 \pm .087$. Wenstrup's experiments were conducted with males from segregating generations after crosses between wild-type stocks Nos. 1 and 11. These males were mated with orange-eyed females of stock No. 3 which was closely related to stock No. 1 but unrelated to stock No. 11. In the selection experiment the material was highly inbred after the initial cross of somewhat related stocks. In the 11-*o* experiment the males were of one stock while the females were grades from an unrelated stock. The significance of the differences is doubtful.

## Reciprocal Matings

It has been shown by Anna R. Whiting (1925) and by Whiting and Anderson (1932) that the production of males among biparentals depends on both parents. To determine whether one parent might have more influence than another on the actual ratio, four sets of reciprocal matings were made (Table VII).

Forty-four matings of cantaloup-eyed females by orange-eyed males from stock No. 3 and 49 reciprocal matings showed an unusually low percentage of males among biparentals. The difference between the two crosses was $1.31 \pm .50$ per cent higher for the orange females by cantaloup males. The percentage of biparentals was higher by 6.3

± 1.7 per cent in the reciprocal. Since the biparental ratio depends so much on the degree of fertilization, even this difference may not indicate any fundamental dissimilarity of the two crosses. Reciprocal matings of cantaloup and ivory-eyed wasps of stock No. 17 gave somewhat similar results. In these crosses biparentals were black-eyed, while azygotic males showed the maternal trait.

A third set of reciprocal matings involved stocks *D-o*, previously described, and reduced (black eyes, reduced wings). *D-o* females by *r* males produced orange azygotic males and black biparentals. Reduced females by *D-o* males gave black reduced azygotic males and black normal-winged biparentals. The percentages were not significantly different for the two crosses.

A fourth set of reciprocal matings involved 11-*o* and reduced. No biparental males were produced although there was some reason to

TABLE VII

*Reciprocal Matings*

| Matings | Matroclinous males | Biparental males | Females | Biparental males × 100 / Biparentals | Biparentals × 100 / Total |
|---|---|---|---|---|---|
| *c* ♀♀ × No. 3 ♂♂..... | 1028 | 3 | 1299 | .23±.13 | 55.9±1.03 |
| No. 3 ♀♀ × *c* ♂♂..... | 658 | 10 | 637 | 1.54±.48 | 49.6±1.4 |
| No. 17 ♀♀ × *c* ♂♂..... | 561 | 8 | 637 | 1.24±.43 | 53.5±1.4 |
| *c* ♀♀ × No. 17 ♂♂..... | 516 | 4 | 718 | .55±.27 | 58.7±1.4 |
| *D-o* ♀♀ × *r* ♂♂..... | 1091 | 43 | 1599 | 2.6 ±.25 | 60.1± .9 |
| *r* ♀♀ × *D-o* ♂♂..... | 546 | 11 | 906 | 1.2 ±.23 | 62.7±1.2 |
| 11-*o* ♀♀ × *r* ♂♂..... | 572 | | 1847 | | 76.4± .8 |
| *r* ♀♀ × 11-*o* ♂♂..... | 342 | | 600 | | 63.7±1.5 |

expect them, since 11-*o* came originally from a cross of stocks 11 and 3, to which latter reduced is related.

The third and fourth sets of matings serve as evidence, in addition to that reported by others, showing that both parents are necessary for the production of males among biparentals. Reduced males crossed to *D-o* females sired biparental sons but failed to sire them when crossed to 11-*o* females. If the reaction depends on the males both crosses should have given biparental sons. On the other hand, reduced females crossed to 11-*o* males gave no biparental sons but did when crossed to *D-o* males. In this case, if the reaction depends on the female, both crosses should have yielded similar results. Thus it is shown that the reaction depends on both parents, and that whatever mechanism causes the production of these anomolous males occurs at fertilization or soon afterwards.

It is of interest to see that in these reciprocal crosses, where the percentage of males among biparentals is very low, the biparental ratio is always less for the cross having the greater proportion of biparental males.

## Fertility of Biparental Males

Anna R. Whiting (1925) and Magnhild M. Torvik (1931) have shown that a large proportion of biparental males are sterile, and that those which are fertile produce very few daughters. E. J. Wenstrup has suggested (unpublished) that biparental males might be more likely to be fertile when they are members of a fraternity having a high ratio of such males. Fifty-seven biparental males, most of which were taken from fraternities having ratios above 25 per cent, were each mated to a virgin female. Each male was tested once, being observed to mate, and then left with the female three or four days. Only four of the males were fertile, and no association between fertility and the ratio of their occurrence was apparent.

## Discussion

The most important aspect of the problem of male biparentalism is the composition or genetic constitution of such males. Knowledge of the varying proportions in which they are produced in different crosses contributes to the solution of this problem and supports the theory of sex-determination recently suggested by P. W. Whiting (1933).

According to this theory females have two sets of autosomal chromosomes, $2A$, and two sex chromosomes, $X$ and $Y$. Azygotic males, developing from unfertilized eggs, are $A + X$ or $A + Y$. Fertilization of an $A + X$ egg nucleus by an $A + Y$ sperm, or the reciprocal, gives rise to a female. In crosses of unrelated stocks no fertilization of $A + Y$ by $A + Y$ or $A + X$ by $A + X$ occurs, but in related stocks such fertilizations may occur, giving rise to biparental males. Haploid males are $A + X$ or $A + Y$, females are $2A + X + Y$, and biparental males are either $2A + 2X$ or $2A + 2Y$.

It may be supposed that crosses of unrelated stocks fail to produce biparental males because of selective fertilization, there being incompatibility between like egg and sperm nuclei. When related stocks are crossed, this incompatibility is reduced.

Data presented in this paper are held to support the view that certain hereditary factors allow the reaction which produces biparental males to take place, and that other factors, of a modifying nature, determine the

percentage of such males among the biparentals. The first group of factors may be thought of as allowing fertilization between like gametes to occur infrequently. Modifying factors may either increase the frequency of like fertilizations, or possibly may render young stages more viable which are developing from such fertilizations.

Some mechanism does reduce the number of biparental offspring from matings producing biparental males. Anna R. Whiting (1925) first reported this fact. It will be recalled that for the 11-*o*, 25-*o*, and 31-*o* experiments a lower ratio of biparentals is typical of fraternities including biparental males, as opposed to fraternities lacking them. This also suggests that fertilization by related sperm tends to prevent the development of biparentals.

In 1923 Anna R. Whiting (Whiting and Anderson, 1932) made daily observations on oviposition and development of progeny of 17 females, which were of several kinds. Females, bred as virgins, crossed to unrelated males, or crossed to related males, laid about the same number of eggs. Hatchability of eggs from the last group was significantly less than from the first two. Fertilization by related sperm was considered to have a lethal effect. In the cross of related stocks, biparental males, if produced, were not detectable.

Selection in several directions, in the selection experiment, indicated that the strain was practically homozygous for factors affecting male biparentalism, after the $F_{21}$ generation. However, in the $F_{29}$ one fraternity out of nine did not contain biparental males, in the $F_{27}$ six of nineteen lacked such males, and in the $F_{25}$ there were seven of forty lacking them. In the $F_{21}$ generation all fraternities (17) included biparental males. Examination of the data for the individual fraternities of generations 25, 27, and 29 reveals that practically all fraternities lacking biparental males had fewer biparentals than several other fraternities which included a single biparental male. For example, in the $F_{27}$ the six fraternities having no biparental males had, respectively, 17, 39, 37, 18, 11, and 20 females. Other fraternities having a single biparental male had, respectively, 41, 56, 43, and 32 females. Thus it is probable that the stock was practically uniform genetically and that it was merely a matter of chance that some fraternities had no biparental males.

No exceptions were found to the rule that unrelated stocks do not produce biparental males. The grading of one stock up to another, as was done in the 11-*o*, 25-*o*, and 31-*o* experiments, was accompanied by the presence of biparental males, and a gradual increase in the incidence of their occurrence. Increasing the degree of relationship between

parental stocks apparently allowed more fertilizations by like sperms. This decrease in the incompatibility between like gametes is probably due to the attainment of homozygosity for more and more factors affecting male biparentalism. The somewhat different results obtained in the four experiments were probably due to the presence of different factors in the wild-type stocks used.

## Summary and Conclusions

1. Selection was effective in increasing the proportion of matings producing biparental males, and in some cases, the proportion of these males in the fraternities including them.

2. Crosses of unrelated stocks do not produce biparental males. When recessive females derived from the $F_1$ generation are mated to the parental stock having the dominant character, biparental males are produced. Subsequent grading up to this parental stock increases the proportion of such males.

3. Hereditary factors determine the production of biparental males and modify the numbers produced.

4. Fraternities containing biparental males have a lower proportion of biparentals than do fraternities lacking such males.

5. There is apparently no association between fertility of biparental males and the incidence of their occurrence.

6. Different inbred wild-type stocks have different hereditary factors affecting male biparentalism.

7. Results are in full agreement with the theory of sex-determination recently proposed by P. W. Whiting.

## Literature Cited

Charles, D. R., 1930. Selection for Biparental Males. *Proc. Penna. Acad. Sci.*, **4**: 85.

Torvik, Magnhild M., 1931. Genetic Evidence for Diploidism of Biparental Males in Habrobracon. *Biol. Bull.*, **61**: 139.

Torvik, Magnhild M., 1933. Chromosomes of Habrobracon. *Proc. Penna. Acad. Sci.*, **7**: 119.

Whiting, Anna R., 1925. The Inheritance of Sterility and of Other Defects Induced by Abnormal Fertilization in the Parasitic Wasp, Habrobracon juglandis (Ashmead). *Genetics*, **10**: 33.

Whiting, Anna R., 1927. Genetic Evidence for Diploid Males in Habrobracon. *Biol. Bull.*, **53**: 438.

Whiting, P. W., 1933. Sex-Determination in Hymenoptera. *Biol. Bull.*, **65**: 357.

Whiting, P. W., and R. L. Anderson, 1932. Temperature and Other Factors Concerned in Male Biparentalism in Habrobracon. *Am. Nat.*, **66**: 420.

# THE CULTIVATION OF A CELLULOSE-DIGESTING FLAGELLATE, TRICHOMONAS TERMOPSIDIS, AND OF CERTAIN OTHER TERMITE PROTOZOA [1]

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## Introduction

The cultivation of some of the intestinal wood-feeding flagellates of termites, known to be necessary for the existence of their host on a cellulose diet (Cleveland, 1924), was attempted with three main purposes in mind. These were: (1) to find what inorganic environment was suitable for these highly specialized protozoa; (2) to obtain in culture for the first time a strictly cellulose-feeding protozoön and to study its nutritional requirements; (3) to discover in what way the action of the protozoa on cellulose furnishes food to their insect host.

For most of the work the large Californian termite, *Termopsis angusticollis,* served as the source of material, although the eastern termite, *Reticulitermes flavipes,* and the roach, *Cryptocercus punctulatus,* were also used. The intestinal fauna of *Termopsis angusticollis* consists of seven species of flagellates of which three, *Trichonympha campanula, T. collaris* and *T. sphærica,* are hypermastigotes, while the other four, *Trichomonas termopsidis, Tricercomitus termopsidis, Hexamastix termopsidis,* and *Streblomastix strix* are polymastigotes. All except the last three feed regularly on cellulose.

## Culture Experiments

By means of preliminary experiments conducted in culture cell slides, I found that the most favorable osmotic pressure for the protozoa of *T. angusticollis* is that of a 0.3 to 0.4 per cent sodium chloride solution, that the favorable pH range is 6.8 to 7.2, that a ratio of 97 equivalents of Na

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ion to three of Ca is an optimum one, and that an excess of Na over K is more favorable than the reverse.

Many balanced salt solutions were made conforming more or less to these requirements. The anions used in most of the solutions were chloride, phosphate, bicarbonate, and citrate. Those solutions in which the protozoa were able to survive in good condition for at least a day were then used, with certain nutrients added, for actual culture experiments.

In the setting up of a culture experiment, test tubes provided with a small amount of powdered cellulose and a little Merck's powdered animal charcoal were used. The cellulose was prepared by dissolving Whatman filter paper No. 40 in Cross and Bevans' reagent (1 part by weight $ZnCl_2$, 2 parts concentrated HCl) and reprecipitating in water. The precipitated cellulose was filtered off on a large Buchner funnel, washed with tap water till free of chloride and then with distilled water, and allowed to air-dry. It could then be easily ground up in a mortar to a fine white powder containing many particles small enough to be ingested even by the smallest cellulose-feeding flagellates. The tubes holding the cellulose and charcoal were sterilized either in the autoclave or by dry heat. Each tube then received approximately 8 cc. of the liquid medium, which had been previously sterilized by filtration through a Berkefeld N filter. The liquid in the tubes was covered with sterile vaseline, which effectively prevented any rise in pH due to the escape of $CO_2$ from the medium.

In most cases, each tube was inoculated with the entire hindgut of a termite. The termite was first disinfected on the outside by a 15 to 40-minute immersion in 1:1000 $HgCl_2$ (this did not injure the protozoa) followed by washing in 95 per cent alcohol and in sterile water. The gut was removed with sterile instruments and placed in the culture medium. The liquid was then again covered with sterile vaseline or left exposed, depending on the particular experiment. The tubes, kept at room temperatures in a large closed cupboard, were examined only once or twice a week, since the growth of the protozoa, if any, was always slow.

By these methods a medium (Solution *A*, Table I) was found in which, with the addition of the proper nutrients, three termite flagellates have been cultured for over three years. Two of these, an as yet undescribed *Trichomonas* from *R. flavipes*, and *Tricercomitus termopsidis*,[3] do not require cellulose. The third, *Trichomonas termopsidis*, must

[3] Full details concerning the cultivation of *Tricercomitus termopsidis* and its method of "encystation" are given in a separate paper to appear in *Arch. f. Protistenkunde*.

have cellulose and small amounts of blood serum. Cultures of this organism were used in several experiments described in the second part of this paper.

Of the hypermastigotes, only *Trichonympha sphærica* was able to survive several days in Solution *A* plus Loeffler's dehydrated blood serum (.001 to .2 per cent) with cellulose and charcoal, but no multiplication took place. A series of changes in the salt composition of Solution *A* led to a solution in which (with .001 per cent Loeffler's blood serum, cellulose, and charcoal) *Trichonympha sphærica* lived several weeks and multiplied slightly. This solution was tried with a variety of nutrients (casein, peptone, amino acids, nucleic acid, etc.) and under a variety of physical conditions (as in collodion bags surrounded by large volumes of sterile culture fluid), but no improvement

TABLE I

| Salt | Grams per liter distilled water | |
|---|---|---|
| | Solution A | Solution U |
| $NaCl$ | 1.169 | 2.164 |
| $NaHCO_3$ | 0.840 | 0.773 |
| $Na_3C_6H_5O_7.2H_2O$ (citrate) | 2.943 | 1.509 |
| $NaH_2PO_4.H_2O$ | 0.690 | 0 |
| $KCl$ | 0.745 | 0 |
| $KH_2PO_4$ | 0 | 1.784 |
| $CaCl_2$ | 0.111 | 0.083 |
| $MgSO_4$ | 0 | 0.048 |

was effected. Further changes in the salt composition finally gave Solution *U* (Table I) in which (with .01 per cent Loeffler's blood serum, cellulose, and charcoal) good initial cultures of *T. sphærica* were obtained. The organisms were usually most abundant two weeks after inoculation of the tube and grew best when the liquid was not covered with vaseline. At the time of maximum population the pH of the cultures was 7.4. After the second week the pH continued to rise, while the number of protozoa decreased slowly, some surviving as long as six weeks. Subcultures, made with 0.5 to 1 cc. of material, showed fair growth in a small percentage of cases, but no second subculture could be effected. Slight changes in the composition of Solution *U* and in its pH, the use of nutrients other than Loeffler's blood serum, and variations in the degree of exposure to air all failed to produce any improvements. Excellent multiplication in many of the initial cultures and in a few of the first subcultures, but never any multiplication in any of the

second subcultures, suggested the possibility that some essential substance present in the termite's gut is either used up or removed by dilution. Accordingly, a number of termites were defaunated by oxygenation (Cleveland, 1925) and a series of first and second subcultures was then made, with each tube receiving a gut aseptically removed from one of the defaunated termites. Few good first subcultures and no second subcultures were obtained.

It is nevertheless interesting that an organism as highly specialized, both morphologically and physiologically, as *T. sphærica* should have been able to live and multiply to a considerable extent in a relatively simple culture medium. It is also noteworthy that in the case of the three polymastigotes as well as in the case of the hypermastigote, the salt composition of the medium is of the utmost importance. Thus *Trichomonas termopsidis* could be cultured continuously only in Solutions *A* and *U* and not in several other very similar media. Moreover, although Solutions *A* and *U* differ from each other only in the presence of $MgSO_4$ and of more phosphate and less citrate in the latter, yet these small differences were sufficient to make all the difference between mere survival of *Trichonympha sphærica* in *A* and active multiplication in *U*.

## Trichomonas termopsidis in Culture; its Nutrition and its Action on Cellulose

The xylophagous flagellate *T. termopsidis* has been maintained in culture since October, 1930, in Solution *A* with 0.2 per cent Loeffler's blood serum, cellulose, and charcoal. The liquid in each tube is kept covered with a layer of vaseline except for a short time when the vaseline seal is broken to permit examination. The pH of the medium, originally 7.0 to 7.2, never goes below 6.8, regardless of the age of the culture.

Subcultures are made from parent cultures two, three, or four weeks old, using about 0.3 cc. of material to a tube. The inoculum is removed from the very bottom of the culture, for the flagellates are rarely present more than a quarter of an inch above the bottom. Before the use of charcoal was begun, growth failed to appear in some of the subcultures, but ever since then the subcultures have been completely successful. In most subcultures growth is slow during the first week, reaching a value of 0.2 to 1 organism per low-power field (10 such fields counted in a sample of one drop from the bottom of the tube). At the end of the second week from 1 to 5 organisms per field are present, and at the end of the third week from 8 to 20. After the third week the cultures will remain in a stationary state for a week or even several weeks, after which time the organisms begin to die off. There were

considerable variations from this usual state of affairs. Some few cultures never got beyond one or two organisms per field, while in others there might be fifty or more.

The *Trichomonas termopsidis* in a culture, up to the time of the fourth or fifth week, are nearly all very active and perfectly normal in appearance, and their bodies contain varying numbers of cellulose particles. Most of the flagellates are from 30 to 50 $\mu$ long, although in very young cultures, where division forms are numerous, the smaller organisms predominate. In some old cultures, giant forms and multiple-fission forms have been encountered which can ingest very large pieces of cellulose. All the cultural forms are so obviously like those seen in termites that no detailed morphological study of them has been made. (For the morphology of *T. termopsidis* see Andrews, 1925, and Kirby, 1931.)

*Trichomonas termopsidis* appears to require very little, if any, oxygen. Not only do the organisms grow best under a vaseline seal, but good growth could also be obtained in tubes connected by means of paraffined stoppers and glass tubing to other tubes containing alkaline pyrogallol, thus creating anærobic conditions. Moreover, exposure of the organisms to atmospheric oxygen is toxic. Thus, when exposed to air in a thin layer of culture fluid in a moist chamber the flagellates rapidly round up and die, while if similarly exposed to nitrogen they continue to swim about actively for several days. A current of air bubbled through a culture at the rate of 60 bubbles per minute killed all the protozoa in less than 24 hours, while a current of nitrogen bubbled through another portion of the same culture at the same rate had no effect.

A bacteriological study of the cultures of *T. termopsidis*, made when the strains were several months old, revealed that only one species of bacteria (derived from the termite's gut) was present. This organism, a short Gram-negative bacillus, grows well at room temperature in broth, as well as in Solution *A* with only $(NH_4)_2SO_4$ as its source of nitrogen. It ferments glucose with gas production, but it does not attack cellulose or cellobiose and cannot live in media ordinarily used for cellulose-fermenting bacteria. Many attempts have been made, using a variety of methods, to free the cultures of this last contaminating organism, but none was successful.

Although the ideal of a bacteria-free culture has thus not been attained, it has nevertheless been possible to perform some experiments concerning the nutrition of *Trichomonas termopsidis*. In one experiment, subcultures were made from healthy and comparable parent cultures growing in the standard medium into media in which the Loeffler's

dehydrated blood serum was replaced by 0.1 per cent Difco desiccated blood serum, 0.1 per cent beef broth, 0.1 per cent Bactopeptone, 0.1 per cent cystine plus 0.2 per cent glycine, 0.01 per cent cystine plus 0.01 per cent glycine plus 0.01 per cent tyrosine, 0.1 per cent asparagin or 0.1 per cent Difco hydrolyzed blood serum. Growth occurred only in media containing blood serum or hydrolyzed blood serum. In other series of experiments the cellulose was replaced by rice starch, dextrin, inulin, glycogen, cellobiose, agar, cellulose tri-acetate, or cellobiose octa-acetate. No growth of the flagellates took place, although controls containing cellulose always showed excellent growth. *Trichomonas termopsidis* in

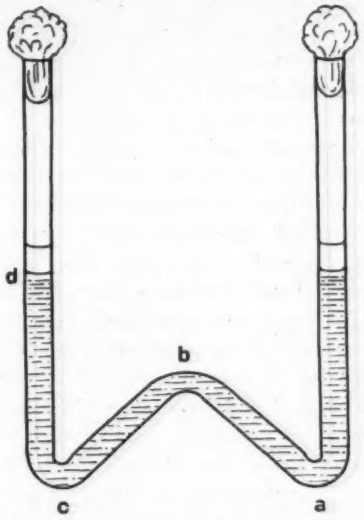


FIG. 1. Diagram of M-tube.

culture appears to be specific for cellulose as its carbon source. In this respect it, like most of the other symbiotic flagellates of termites, closely resembles certain bacteria, such as *Spirochæta cytophaga* (Hutchinson and Clayton, 1919) and *Bacillus cellulosæ-dissolvans* (Khouvine, 1923), which are likewise limited to cellulose as their carbon source.

The maintenance of *Trichomonas termopsidis* for several years on a cellulose diet, its inability to live in culture when the cellulose is replaced by other polysaccharides, and the extraction from the cultivated organisms of a cellulase (Trager, 1932) prove that this flagellate is capable of digesting cellulose.

Since the bacillus present in the cultures of *Trichomonas termopsidis* is a very active glucose fermenter, no hope was entertained of being able to demonstrate unequivocally the production of glucose by cultures of the flagellate. Tests with Benedict's solution of the fluid of active cultures were always negative. Some very strong indirect evidence for glucose production was, however, obtained. If an active culture of the trichomonads was divided into two equal parts, of which one was heated to 43° and held there for 10 minutes, thus killing all the protozoa without injuring the bacteria, and if each portion was then placed in a fermentation tube or in a test tube with a heavy layer of vaseline over it, then, after the lapse of several days, gas appeared only in the tube with the live protozoa. To determine whether this gas was produced by the bacteria from glucose formed through the activities of the protozoa, or whether it was produced directly by the protozoa, the following experiment was performed. Twenty active cultures of *T. termopsidis* were mixed in a large sterile tube and centrifuged. The supernatant liquid, determined by microscopic examination to be free of protozoa, was removed completely, and then the residue, containing the protozoa, was resuspended in 5 cc. of this supernatant liquid. Four tubes, shaped like inverted M's (Fig. 1), made from 6 mm. glass tubing and previously plugged with cotton and sterilized, were now set up as follows. Numbers 1 and 2 received each two pipettefuls, and Nos. 3 and 4 three pipettefuls, of the protozoa-free supernatant liquid. The tubes were manipulated so that no air-bubbles remained in them. To one arm, suitably marked, of Nos. 1 and 3 was then added one pipetteful of the protozoan suspension, very carefully and in such a manner that the protozoa settled in notch *a* of the tube and did not rise to the level of notch *b* (Fig. 1). The remainder of the protozoan suspension was then heated to 43° C. and kept at this temperature for 10 minutes. One pipetteful of this dead suspension was then added to one arm of Nos. 2 and 4. The liquid in both arms of all four tubes was covered with a heavy layer of vaseline. In such an arrangement, it is evident that most of the gas formed should accumulate at notch *b* and that, if the protozoa cannot swim up over notch *b*, then any gas appearing in notch *c* or under the vaseline at *d* (Fig. 1) could not possibly have been formed by the protozoa. Within one week after the date of setting up the experiment, in both tubes 1 and 3 gas was present at notch *c* as well as at notches *a* and *b*. Microscopic examination of samples removed from notches *a* and *c* showed that protozoa were present only in notch *a*. In tubes 2 and 4 there was no gas whatever. This proves conclusively that, in

tubes 1 and 3, the protozoa present in notch *a* produced by their action on cellulose a soluble substance which diffused over toward notch *c* and was there fermented by the bacteria. That this substance was glucose seems almost certain, in view of the facts that glucose and cellobiose are the only simple sugars formed from cellulose and that the bacillus present readily ferments glucose with gas production, but cannot utilize cellobiose. That this glucose was not produced as the result of the extra-cellular action of cellulase is indicated by the fact that, although cellulase could be demonstrated in the supernatant fluid of old degenerating cultures, it could not be demonstrated in the supernatant fluid of active healthy cultures such as were used for the experiment detailed above. It thus appears that *T. termopsidis* and other symbiotic intestinal flagellates ingest cellulose, digest it to glucose, and then excrete part of the glucose, sharing it with their insect host.

## Summary

By means of preliminary experiments, the osmotic pressure, pH, and mono- to bivalent ion ratio most favorable to the survival of the symbiotic intestinal flagellates of termites were determined. On the basis of these and other facts, balanced salt solutions were constructed and tested, with the addition of cellulose and low concentrations of protein, as culture media.

In this way a medium was obtained in which the xylophagous flagellate, *Trichomonas termopsidis,* from *Termopsis angusticollis,* has been cultured for over three years. A *Trichomonas* from *Reticulitermes flavipes,* and *Tricercomitus termopsidis* from *Termopsis angusticollis* were cultured in the same medium. The last two organisms did not require cellulose.

In a somewhat different medium, excellent initial cultures of the hypermastigote, *Trichonympha sphærica,* from *Termopsis angusticollis,* were obtained, and the organisms could be carried through a first subculture but not through a second. Attempts to improve the medium so as to secure continuous cultivation failed.

Experiments with *Trichomonas termopsidis* showed that it cannot utilize any carbon source other than cellulose. This protozoön probably secretes glucose, which is used by its insect host.

## References

Andrews, J. M., 1925. Morphology and Mitosis in *Trichomonas termopsidis,* an Intestinal Flagellate of the Termite, Termopsis. *Biol. Bull.,* **49**: 69.

Cleveland, L. R., 1924. The Physiological and Symbiotic Relationships between the Intestinal Protozoa of Termites and their Host, with Special Reference to *Reticulitermes flavipes* Kollar. *Biol. Bull.,* **46**: 178.

CLEVELAND, L. R., 1925. Toxicity of Oxygen for Protozoa in vivo and in vitro: Animals Defaunated without Injury. *Biol. Bull.*, **48**: 455.

HUTCHINSON, H. B., AND J. O. CLAYTON, 1919. On the Decomposition of Cellulose by an Aerobic Organism. *Jour. Agric. Sci.*, **9**: 143.

KHOUVINE, Y., 1923. Digestion de la cellulose par la flore intestinale de l'homme. *Ann. Past.*, **37**: 711.

KIRBY, H., 1931. Trichomonad Flagellates from Termites. II. *Univ. Calif. Publ. Zoöl.*, **36**: 171.

TRAGER, W., 1932. A Cellulase from the Symbiotic Intestinal Flagellates of Termites and of the Roach, Cryptocercus punctulatus. *Biochem. Jour.*, **26**: 1762.

# THE SEDIMENTATION CONSTANTS OF THE RESPIRATORY PROTEINS

THE SVEDBERG AND ASTRID HEDENIUS

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The ultracentrifugal technique developed in this laboratory enables us to define the mass and shape properties of a high molecular substance by means of two data, the molecular weight and the sedimentation constant (Svedberg, 1934). The latter one, which is a function of the mass and shape of the molecule, is the easiest to measure with a fairly high degree of precision. The error in the mean value from a few—say ten—absolute determinations is about 1 per cent. Relative measurements can be arranged so as to give an accuracy of around 2 per cent in a single experiment. The amount of substance required for a determination is of the order of 0.001 gram. If the molecules to be studied possess characteristic light absorption in the visible or ultraviolet part of the spectrum, it is possible to make the measurement regardless of the presence of other substances in the solution. In many cases the method further allows us to measure simultaneously the sedimentation constants of the different molecular species in a mixed solution. The determination of the sedimentation constant therefore constitutes an efficient tool for the study of high molecular substances dissolved in native fluids. On the following pages a report will be given of an attempt to characterize the respiratory proteins throughout the animal kingdom by their sedimentation constants (see Svedberg and Hedenius, 1933; Svedberg, 1933).

## Method

A small quantity of the solution to be studied (0.1 to 1.5 cc.), enclosed in a sector-shaped cell between plane-parallel windows of crystalline quartz, is exposed to the influence of a very strong field of force in a special centrifugal instrument, the ultracentrifuge. In order to avoid convection currents due to temperature disturbances, the rotation takes place in hydrogen of 20 mm. pressure and the surface of the solution is covered with a layer of oil. From time to time photographs of the rotating column of solution are taken so as to record the movement of the boundary between solvent and sedimenting solute. A concentration scale is photographed on the same plate. The pictures are then reg-

istered by means of a microphotometer and the curves obtained used for the calculation. If the field of force is strong enough, the rate of settling can be measured and from this the sedimentation constant, defined as the velocity of sedimentation in a field of unit strength reduced to water of 20° as solvent, can be calculated.

If $x$ is the distance from the centre of rotation, $t$ the time, $\omega$ the angular velocity, $\eta$ the viscosity and $\rho$ the density of the solvent, $\eta_0$ the viscosity and $\rho_0$ the density of water of 20°, and $V$ the partial specific volume of the solute, the sedimentation constant is given by the expression:

$$s_{20}^{\circ} = dx/dt \,.\, 1/\omega^2 x \,.\, \eta/\eta_0 \cdot \frac{1 - V\rho_0}{1 - V\rho}.$$

From previous protein investigations of this laboratory it is known that $V$ varies very little from one protein to the other. The fact that $V$ only figures in a correction term makes it unnecessary to determine it for every protein studied. This circumstance is of considerable importance because of the difficulty of procuring enough material for density determinations in the case of many of the invertebrates. In the following the mean value $V = 0.745$ was used.

The active group of the respiratory pigments confers upon these proteins characteristic absorption bands in the visible and near ultraviolet spectrum. If a suitable wave-length is chosen for the illumination, it is therefore always possible to measure the sedimentation of the molecules of a respiratory pigment undisturbed by the presence of other blood proteins. A sample of blood can be studied in the ultracentrifuge directly after drawing it and eventually diluting it with a salt solution (or laking the blood corpuscles) without subjecting it to any chemical treatment at all. This simplified procedure eliminates serious causes of error in the case of the more unstable pigments.

Four types of respiratory blood proteins are known, red pigments (erythrocruorin, hemoglobin), green pigments (chlorocruorin), blue pigments (hemocyanin) and pigments of reddish-brown color (hemerythrin) (Redfield, 1933). As a rule we have used the mercury lines 577–79 mμ and 546 mμ in combination with a Wratten K3 filter for the study of the red and green proteins, the mercury line 366 mμ with a nickel oxide glass filter for the hemocyanins as well as for mixtures of red or green proteins with hemocyanins and line 546 mμ with a Wratten n:r 77 filter for the hemerythrins. The pictures in the visible spectrum have been taken on Ilford rapid process panchromatic plates while for the ultraviolet we have used Imperial process plates.

For those species the blood of which contains a respiratory protein

of high molecular weight at least two determinations have been made, one for the blood in question alone, and one in mixture with the blood of a standard species. This last kind of measurement enables us to compare all the sedimentation determinations with a few well-established constants. The absence of a double boundary in the case of a mixture of two proteins of high molecular weight, and accordingly low diffusion, has been taken as a criterion of the identity of the sedimentation constants (limit of error about $\pm$ 2 per cent). On the other hand, the existence of two different sedimentation constants lying close together has always been checked by applying this mixture test.

The blood samples have mostly been diluted down to a pigment concentration of 0.1–0.3 per cent in order to avoid viscosity errors. A few check runs on undiluted blood have been done, however. As a rule we have used 1 per cent NaCl as diluting agent. The natural pH of the blood is often very close to the alkaline stability limit of the respiratory protein contained in it. In such cases, where more than one kind of molecular mass exists at this border, the samples have been diluted with a suitable buffer solution to keep the pH down. The sedimentation constant of the state of aggregation which possesses the widest pH stability range has always been chosen to characterize the protein in question. The sedimentation constants of dissociation or aggregation states observed have been given in parenthesis in the tables.

Most of the invertebrates have their blood pigment dissolved in the plasma. The echinoderms, the lamellibranchians, some of the worms and, of course, all the vertebrates, however, have red blood corpuscles. To bring the pigment into solution the erythrocytes were laked with distilled water and filtered off. Sodium chloride was then added to bring the salt content up to 1 per cent. In cases where we had at our disposal a quantity of blood sufficient for separation of the pigment from colorless proteins eventually present the corpuscles were washed on the centrifuge with sea water or with 1 per cent NaCl solution before laking.

It was found during this investigation that the blood samples obtained from invertebrate animals were often apt to deteriorate rapidly even when kept at 0° C. This was especially pronounced in the case of very small species where it was impossible to avoid contamination of the blood by coelomic fluid or gland excretions. At least one run for each species was therefore carried out immediately after drawing the blood. Tests with samples of frozen blood, however, showed that even the most unstable types could be stored in this state for months without deteriorating. During the latter part of this investigation constant use was made of this circumstance.

TABLE I

Chætopoda: Polychæta, Hirudinea

Pigment dissolved in plasma. Standard species for mixture test, *Nereis virens* (Sars). Solvent, 1 per cent NaCl; thickness of column of solution 1.2 cm.; centrifugal force about 65,000 times gravity.

| | Origin of blood | Dilution of blood | Mixed with blood of | $s_{20}° \times 10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|---|
| | | | | | *per cent* |
| Type of pigment: erythrocruorin | Eunicidæ: Lumbrinereis fragilis (Müll.) Kristineberg, Sweden | 10 | | 58.0 | – |
| | | 20 | Nereis virens | 59.8† | ±3 |
| | Nereidæ: Nereis virens (Sars) Kristineberg, Sweden | 10 | | 58.6* | – |
| | | 20 | | 61.5* | – |
| | | 15 | | 56.6 | – |
| | | 30 | | 57.6‖ | – |
| | | 20 | Lumbrinereis fragilis | 61.5† | – |
| | | 20 | Arenicola marina | 57.0* | – |
| | | 15 | Eumenia crassa | 59.0 | – |
| | | 20 | Pectinaria belgica | 54.1 | – |
| | | 13 | Polymnia nebulosa | 59.1 | – |
| | | 20 | Sabella pavonia | 53.8 | – |
| | | 20 | Hæmopis sanguisuga | 57.6 | – |
| | | 20 | Hirudo medicinalis | 58.5 | – |
| | | 20 | Lumbricus terrestris | 58.4* | – |
| | | 10 | Planorbis corneus | 56.2 | – |
| | | 20 | Helix pomatia | 59.6§ | – |
| | | 20 | Sepia officinalis | 58.9 | – |
| | Arenicolidæ: Arenicola marina (L.) Kristineberg, Sweden | 20 | Nereis virens | 57.8* | ±1 |
| | Scalibregmidæ: Eumenia crassa Oersted Kristineberg, Sweden | 10 | | 61.4 | – |
| | | 10 | Nereis virens | 57.3 | ±3 |

TABLE I—*Continued*

TABLE I—*Continued*

| | Origin of blood | Dilution of blood | Mixed with blood of | $s_{18}^{\circ} \times 10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|---|
| | | | | | †*per cent* |
| Type of pigment: erythrocruorin | Amphictenidæ: Pectinaria belgica (Pall.) Kristineberg, Sweden | 5 | | 54.6(11.7) | – |
| | | 20 | Nereis virens | 54.3(11.7) | ±0 |
| | Terebellidæ: Polymnia nebulosa (Mont.) Kristineberg, Sweden | 2 | | 56.4 | – |
| | | 2 | Nereis virens | 58.3 | ±1 |
| Type of pigment: chlorocruorin | Chlorhæmidæ: Brada villosa Rathke Kristineberg, Sweden | — | | 56.0‡ | |
| | | 2 | | 56.3‡ | |
| | Serpulidæ: Serpula vermicularis L. Kristineberg, Sweden | 4 | | 58.8 | |
| | Sabella pavonia Sav. Kristineberg, Sweden | 8 | | 52.7 | |
| | | 6 | Nereis virens | 54.4 | ±1 |
| Type of pigment: erythrocruorin | Hirudinidæ: Hirudo medicinalis L. South Europe | 25 | | 58.5 | – |
| | | 25 | Nereis virens | 57.5 | ±1 |
| | Hæmopis sanguisuga L. Upsala, Sweden | 35 | | 57.7 | – |
| | | 35 | Nereis virens | 56.5 | ±2 |
| | | | Mean | 57.5 | ±1.5 |

* Thickness of column of solution 0.8 cm.
† Thickness of column of solution 1.0 cm.
‡ Thickness of column of solution 0.2 cm.; mercury lines 405–436 mμ; Wratten nr 50 filter, Imperial Process plates.
§ Centrifugal force 39,000 times gravity.
|| Centrifugal force 21,000 times gravity; blood frozen for six months, determined by Mrs. I.-B. Eriksson-Quensel.

The following buffer solutions were used:

pH 5.0. 0.0059 M acetic acid, 0.0141 M Na-acetate, 0.8 per cent NaCl.
pH 6.8. 0.01 M $Na_2HPO_4$, 0.01 M $KH_2PO_4$, 0.1 M KCl.
pH 8.0. 0.019 M $Na_2HPO_4$, 0.001 M $KH_2PO_4$, 0.1 M KCl.

TABLE II

Chætopoda: Polychæta

Pigment enclosed in corpuscles. Type of pigment, erythrocruorin; solvent, 1 per cent NaCl; thickness of column of solution, 1.2 cm.; centrifugal force about 180,000 times gravity.

| Origin of blood | $s_{20}° \times 10^{13}$ |
|---|---|
| Glyceridæ: | |
| Glycera Rouxii Aud. and M. E. | |
| Kristineberg, Sweden | 3.56 |
| | 3.44 |
| Mean | 3.50 |
| Capitellidæ: | |
| Notomastus latericeus Sars | |
| Kristineberg, Sweden | 2.18 |
| | 1.98 |
| Mean | 2.08 |

TABLE III

Chætopoda: Oligochæta

Pigment dissolved in plasma. Type of pigment, erythrocruorin. Standard species for mixture test, *Lumbricus terrestris* L. Solvent, 1 per cent NaCl; thickness of column of solution 1.2 cm. Centrifugal force about 65,000 times gravity.

| Origin of blood | Dilution of blood | Mixed with blood of | $s_{20}° \times 10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|
| | | | | *per cent* |
| Lumbricidæ: | | | | |
| Lumbricus terrestris L. | | | | |
| Upsala, Sweden | 20 | | 60.6 | — |
| | 20 | | 60.0* | |
| | 20 | | 63.1† | — |
| | 20 | Nereis virens | 63.0‡ | — |
| | 20 | Tonicella marmorea | 62.1 | — |
| | 40 | Sepia officinalis | 59.9 | — |
| | 30 | Eisenia fœtida | 65.6 | — |
| | 20 | Arion ater | 57.7 | — |
| Eisenia fœtida Sav. | | | | |
| Upsala, Sweden | 20 | | 63.5 | |
| | 30 | Lumbricus terrestris | 63.4 | ±4 |
| | | Mean | 61.9 | |

* Blood frozen for 16 days.
† Blood frozen for 49 days.
‡ Thickness of column of solution, 0.8 cm.

In the tables they will be denoted by their pH. The additions of neutral salt have been made in order to repress the disturbances of the sedimentation caused by the electric charge of the protein molecules (Donnan effect).

## Sedimentation Constants

### *Scolecida*

Among the lower worms one representative of the nemerteans was examined, but it was found impossible to draw enough blood from it for a determination of the sedimentation constant.

### *Annelida*

A considerable number of species belonging to this group have been studied. In most cases the respiratory pigment is in solution and sufficient quantities of blood may easily be drawn. A large worm like *Arenicola* may yield up to 0.3 cc., while it requires about a dozen individuals of a small species like *Serpula* to make up 0.1 cc. *Nephthys ciliata, Panthalis oerstedi, Thelepus cincinnatus* and *Terebellides stroemi* were tried without success. Worms with red blood corpuscles are scarce and it is not always easy to extract the pigment without injuring it. With *Echiurus pallasi* and *Priapulus caudatus* we were unsuccessful. Erythrocruorin, chlorocruorin, and hemerythrin but not hemocyanin have been found within this group of animals.

Tables I–III contain the determinations of sedimentation constants. By adopting the mixture test we were able to prove that the dissolved erythrocruorin and chlorocruorin of all the Polychæta and Hirudinea species have the same sedimentation constant with a mean value of $57.5 \times 10^{-13}$. The Oligochæta have a slightly higher constant, viz., $61.9 \times 10^{-13}$. Two different and very low constants, 3.50 and 2.08 $\times 10^{-13}$, were found for the erythrocruorin contained in the corpuscles of the glyceride and capitellide worms. The hemerythrin from the corpuscles of the sipunculoideans has also a low constant, but owing to the scarcity of the material at our disposal (*Phascolosoma vulgare*), accurate values for the sedimentation constant could not be obtained. We have therefore refrained from including these data in the tables. The erythrocruorin of *Pectinaria* is very unstable and decomposes easily, giving rise to a product of sedimentation constant $11.7 \times 10^{-13}$. A dissociation product of the same constant has been observed at the alkaline border of the stability range in the case of *Arenicola* erythrocruorin (Svedberg and Eriksson-Quensel, 1933). Sedimentation equilibrium measurements indicate that the appearance of this product represents the breaking up of the molecule into 16 equal parts of weight 207,000.

## TABLE IV

Crustacea: Phyllopoda, Malacostraca

Pigment dissolved in plasma. Standard species for mixture test, *Pandalus borealis* Krøyer. Solvent, 1 per cent NaCl or buffer pH 5. Thickness of column of solution 1.2 cm.; centrifugal force, 90,000–165,000 times gravity.

| Origin of blood | | Dilution of blood | Mixed with blood of | $s_{20°} \times 10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|---|
| | | | | | *per cent* |
| Type of pigment: erythrocruorin | Daphnidæ: Daphnia pulex de Geer<br>Upsala, Sweden | | | 16.5* †<br>17.2*<br>17.1* | |
| | | | Palinurus vulgaris | 16.8* | |
| | Bopyridæ: Athelges sp.<br>Kristineberg, Sweden | 7 | Planorbis corneus | 19.3 | |
| Type of pigment: hemocyanin | Caridídæ:<br>Pandalus borealis Krøyer<br>Kristineberg, Sweden | 5 | | 16.6 | |
| | | 5 | Palinurus vulgaris | 16.8 | |
| | | 5 | Pagurus striatus | 18.1 | |
| | | 5 | Homarus vulgaris | 17.2 | |
| | Palæmon fabrici Rathke | 15 | Homarus vulgaris | 15.6 | |

TABLE IV—*Continued*

| | Origin of blood | Dilution of blood | Mixed with blood of | $s_{20}^{*} \times 10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|---|
| | | | | | *per cent* |
| Type of pigment: hemocyanin | Palinuridæ: Palinurus vulgaris Latr. | | | | |
| | Naples, Italy | 8 | | 17.2 | |
| | | 9 | | 15.3 | |
| | | 8 | Pandalus borealis | 15.8 | ±6 |
| | | 10 | Daphnia pulex | 18.1 | |
| | Paguridæ: | | | | |
| | Pagurus striatus Latr. | | | | |
| | Naples, Italy | 7 | | 16.0‡ | |
| | | 10 | Pandalus borealis | 16.7‡ | ±8 |
| | Eupagurus bernhardus (L.) | | | | |
| | Kristineberg, Sweden | 5 | Homarus vulgaris | 17.1‡ | |
| | | 5 | | 16.2‡(21.6) | |
| | | 5 | | 17.4‡(22.5) | |
| | | | Mean | 16.9 (22.1) | |

* Thickness of column of solution 0.8 cm.
† Centrifugal force 45,000 times gravity.
‡ Solvent buffer pH 5.

## TABLE V

Crustacea: Malacostraca

Pigment dissolved in plasma. Type of pigment, hemocyanin. Standard species for mixture test, *Homarus vulgaris* M. E. Solvent, 1 per cent NaCl or buffer pH 5. Thickness of column of solution 1.2 cm. Centrifugal force 67,000–150,000 times gravity.

| Origin of blood | Dilution of blood | Mixed with blood of | $s_{20}° \times 10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|
| | | | | *per cent* |
| Squillidæ: | | | | |
| Squilla mantis Latr. | | | | |
| Naples, Italy..... | 10 | | 23.7† | |
| | 10 | Homarus vulgaris | 23.4† | ±3 |
| Nephropsidæ: | | | | |
| Nephrops norvegicus (L.) | | | | |
| Kristineberg, Sweden..... | 8 | | 24.4 | |
| | 8 | Homarus vulgaris | 24.7 | ±1 |
| Homarus vulgaris M. E. | | | | |
| Havstenssund, Sweden..... | 5 | | 24.6* | |
| | 10 | | 24.3† | |
| | 10 | Pandalus borealis | 24.2 | |
| | 10 | Palæmon fabrici | 21.7 | |
| | 10 | Eupagurus bernhardus | 22.1† | |
| | 6 | Cancer pagurus | 22.4 | |
| | 10 | Carcinus mænas | 22.3† | |
| | 6 | Astacus fluviatilis | 23.7 | |
| | 10 | Hyas araneus | 22.3† | |
| | 8 | Nephrops norvegicus | 24.4 | |
| | 10 | Squilla mantis | 22.7† | |
| | 10 | Calocaris macandreæ | 22.6 | |
| | 7 | Planorbis corneus | 23.9 | |
| | 10 | Planorbis corneus | 25.3 | |
| | 10 | Planorbis corneus | 24.8 | |
| | 10 | Sepia officinalis | 23.0 | |
| | 12 | Octopus vulgaris | 22.0 | |
| | 5 | Chiridothea entomon | 22.5 | |
| Astacus fluviatilis L. | | | | |
| Dådran, Sweden... | 5 | | 23.8 | |
| | 10 | | 25.0 | |
| | 10 | | 24.1‡ | |
| | 6 | Homarus vulgaris | 23.4 | ±1 |

TABLE V—*Continued*

| Origin of blood | Dilution of blood | Mixed with blood of | $s_{20}° \times 10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|
| | | | | *per cent* |
| Majidæ: | | | | |
| Hyas araneus (L.) | | | | |
| Kristineberg, Sweden | 6 | | 24.2† | |
| | 5 | Homarus vulgaris | 22.2† | |
| Maja squinado Latr. | | | | |
| Naples, Italy | 1 | | 26.7 | |
| Cancridæ: | | | | |
| Cancer pagurus L. | | | | |
| Havstenssund, Sweden | 5 | | 23.0(15.7) | — |
| | 5 | Homarus vulgaris | 22.5† | ±0 |
| Carcinus maenas (L.) | | | | |
| Kristineberg, Sweden | 6 | | 24.1†(16.4) | |
| | 5 | Homarus vulgaris | 22.2† | ±0 |
| Idotheidæ: | | | | |
| Chiridothea entomon L. | | | | |
| Härnösand, Sweden | 10 | | 23.0(16.1) | |
| | 4 | Homarus vulgaris | 22.7†(16.0) | ±1 |
| | | Mean | 23.5(16.1) | ±1 |

* Thickness of column of solution 1.0 cm.
† Solvent buffer pH 5.
‡ Thickness of column of solution 0.8 cm.

### *Arthropoda*

Among the numerous species of this group possessing respiratory blood proteins there are several large forms, such as the lobster and the horseshoe crab, which yield large quantities of blood. In the case of *Balanus* and *Branchipus* we were unable to collect enough respiratory pigment for a determination of the sedimentation constant. It is doubtful whether the former has any respiratory pigment at all. Species with blood corpuscles are not to be found among the Arthropoda, all species belonging to this group having their pigment dissolved in the blood plasma. Hemocyanin is the commonest pigment; erythrocruorin is also represented; but chlorocruorin and hemerythrin do not occur here. Tables IV–VII contain the determinations of sedimentation constants.

As shown by the mixture test, five different sedimentation constants

were found within this group, viz., 17, 24, $34 \times 10^{-13}$ for the crustaceans; 17, 34, $57 \times 10^{-13}$ for the xiphosurans; $34 \times 10^{-13}$ for the scorpionideans, and the very low constant $2.0 \times 10^{-13}$ for the insects (*Chironomus* larvæ).

The crustacean hemocyanin of constant $23.5 \times 10^{-13}$ gives a dissociation product of constant $16.1 \times 10^{-13}$ at the alkaline border of the stability range. The natural pH of the blood is such that this com-

TABLE VI

Crustacea: Malacostraca;
Arachnomorpha: Xiphosura, Scorpionidea

Pigment dissolved in plasma. Type of pigment, hemocyanin. Solvent, 1 per cent NaCl; centrifugal force about 115,000 times gravity. Thickness of column of solution 1.2 cm.

| Origin of blood | Dilution of blood | Mixed with blood of | $s_{20°} \times 10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|
| | | | | *per cent* |
| Thalassinidæ: | | | | |
| Calocaris macandreæ Bell | | | | |
| Kristineberg, Sweden | 4 | | 34.0 | |
| | 4 | Homarus vulgaris | 33.9 | |
| | 4 | Planorbis corneus | 33.7 | ±4 |
| Limulidæ: | | | | |
| Limulus polyphemus (L.) | | | | |
| Woods Hole, Mass. | 5 | | 34.1(61.2)(16.7) | |
| | 4 | | 33.6*(56.9)(16.3) | |
| Chactidæ: | | | | |
| Euscorpius carpaticus (L.) | | | | |
| Naples, Italy | 11 | | 36.6 | |
| | 11 | Planorbis corneus | 31.9 | ±5 |
| | | Mean | 34.0 | |

* Solvent buffer pH 5.

ponent is almost always observed in the fresh blood. With time the pH decreases spontaneously and the dissociation goes back reversibly. The appearance of this product probably means the breaking up of the molecule into two equal parts. As shown by Table IV, the molecule of constant 16.9 is the normal one in a number of crustacean species both in the case of hemocyanin and erythrocruorin.

TABLE VII

Eutracheata: Diptera

Pigment dissolved in plasma. Type of pigment, erythrocruorin. Solvent, 1 per cent NaCl. Centrifugal force about 216,000 times gravity. Thickness of column of solution 0.8 cm.

| Origin of blood | Dilution of blood | Mixed with blood of | $s_{20°} \times 10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|
| Chironomidæ: | | | | |
| Chironomus plumosus (larvæ) L. | | | | |
| Storvik, Sweden | 20 | | 1.8 | |
| | 20 | | 2.1 | |
| Chironomus sp. | | | | |
| Upsala, Sweden | 12 | | 2.0 | |
| | 12 | | 1.9 | |
| | 8 | | 2.1 | |
| | 8 | | 1.8* | |
| | | Mean | 2.0 | |

* Solvent buffer pH 8.

*Limulus* presents the unique example of a species the blood of which contains hemocyanin of three different sedimentation constants not only at the alkaline border but also near the isoelectric point. A detailed investigation of the *Limulus* blood by Mrs. I.-B. Eriksson-Quensel has shown that these components exist throughout the whole stability

TABLE VIII

Amphineura: Placophora

Pigment dissolved in plasma. Type of pigment, hemocyanin. Solvent, 1 per cent NaCl. Thickness of column of solution 1.2 cm. Centrifugal force about 65,000 times gravity.

| Origin of blood | Dilution of blood | Mixed with blood of | $s_{20°} \times 10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|
| | | | | *per cent* |
| Chitonidæ: | | | | |
| Tonicella marmorea Fabr. | | | | |
| Kristineberg, Sweden | 2.0 | | 60.4 | |
| | 2.5 | | 62.8* | |
| | 2.5 | Lumbricus terrestris | 59.4 | ±5 |
| | | Mean | 60.9 | |

* Centrifugal force 32,000 times gravity.

range. At the acid border the two highest components disappear, giving rise to a new component of constant $23 \times 10^{-13}$. According to determinations by K. O. Pedersen, all the components have the same isoelectric point.

*Mollusca*

From some of the big forms of this group it is easy to draw large amounts of blood in a very pure state. Thus a single individual of the tropical snail *Achatina fulva* yields 50 cc. of blood and a medium-sized

TABLE IX

Conchifera: Gastropoda

Pigment dissolved in plasma. Type of pigment, erythrocruorin. Solvent, 1 per cent NaCl; thickness of column of solution 1.2 cm. Standard species for mixture test, *Planorbis corneus* (L.); centrifugal force 65,000–115,000 times gravity.

| Origin of blood | Dilution of blood | Mixed with blood of | $s_{20}^{0} \times 10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|
| Limnæidæ: | | | | |
| Planorbis corneus (L.) | | | | |
| Upsala, Sweden .... | 10 | | 35.1* † | |
| | 10 | | 31.4* | |
| | 15 | | 33.8 | |
| | 10 | Nereis virens | 33.6 | |
| | 10 | Athelges sp. | 34.0 | |
| | 12 | Homarus vulgaris | 32.2 | |
| | 15 | Homarus vulgaris | 34.2 | |
| | 15 | Homarus vulgaris | 32.4 | |
| | 15 | Calocaris macandreæ | 32.2 | |
| | 15 | Euscorpius carpathicus | 30.5 | |
| | 12 | Helix pomatia | 33.7† | |
| | 15 | Helix pomatia | 35.7† | |
| | 15 | Octopus vulgaris | 33.9 | |
| | 20 | Planorbis umbilicatus | 32.2 | |
| Planorbis umbilicatus (Müll.) | | | | |
| Upsala, Sweden .... | 10 | | 36.7* | |
| | 10 | | 36.9* | |
| | 10 | | 37.2* | |
| | 10 | | 32.9* | |
| | | Mean | 33.8 | |

* Thickness of column of solution 0.8 cm.
† Centrifugal force about 41,000 times gravity.

*Octopus* or *Eledone* gives as much. On the other hand, there are small forms from which we were unable to get pure blood. This was the case with *Dentalium entale*. Other, somewhat larger, forms such as *Æolis*

TABLE X

Conchifera: Gastropoda

Pigment dissolved in plasma. Type of pigment, hemocyanin. Solvent 1 per cent NaCl; thickness of column of solution 1.2 cm. Standard species for mixture test, *Helix pomatia* L.; centrifugal force 22,000–60,000 times gravity.

| Origin of blood | Dilution of blood | Mixed with blood of | $s_{20}° \times 10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|
| | | | | *per cent* |
| Paludinidæ: | | | | |
| Paludina vivipara (L.) | | | | |
| Upsala, Sweden | 10 | | 93.0 | |
| | 20 | | 99.2* | |
| | undil. | | 97.4‡ | |
| Paludina contecta, Millet | | | | |
| Germany | 4 | | 99.6† | |
| | 4 | Helix pomatia | 100.3 | ±2 |
| Littorinidæ: | | | | |
| Littorina littorea (L.) | | | | |
| Kristineberg, Sweden | 10 | | 99.1*(137.0) | |
| | 2 | | 97.3*(126.8) | |
| | 4 | Helix pomatia | 96.3 | ±1 |
| Fasciolaridæ: | | | | |
| Neptunea antiqua (L.) | | | | |
| Kristineberg, Sweden | 15 | | 107.3† | |
| | 20 | Helix pomatia | 107.7† | ±8 |
| Buccinidæ: | | | | |
| Buccinum undatum L. | | | | |
| Kristineberg, Sweden | 8 | | 101.4*(131.6) | |
| | 4 | Helix pomatia | 101.9 | ±1 |
| Turbinellidæ: | | | | |
| Busycon canaliculatum (L.) | | | | |
| Woods Hole, Mass. | 20 | | 101.3(138.8) | |
| | 20 | Helix pomatia | 96.9 | ±0 |

TABLE X—*Continued*

| Origin of blood | Dilution of blood | Mixed with blood of | $s_{20}° \times 10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|
| | | | | *per cent* |
| Limnæidæ: | | | | |
| Limnæa stagnalis (L.) | | | | |
| Upsala, Sweden | undil. | | 94.4 | |
| | " | | 104.1(60.2) | |
| | " | | 96.8 | |
| | " | Helix pomatia | 96.6 | ±3 |
| Pupillidæ: | | | | |
| Achatina fulva Brug. | | | | |
| Africa | 10 | | 95.7(63.1, 15.1) | |
| | 10 | Helix pomatia | 107.4(64.3, 17.3) | ±1 |
| Helicidæ: | | | | |
| Helix pomatia L. | | | | |
| Upsala, Sweden | 12 | Paludina contecta | 102.3 | |
| | 9 | Littorina littorea | 95.4 | |
| | 12 | Neptunea antiqua | 99.5 | |
| | 9 | Buccinum undatum | 100.8 | |
| | 20 | Busycon canaliculatum | 96.6 | |
| | 15 | Limnæa stagnalis | 99.2 | |
| | 9 | Achatina fulva | 105.7 | |
| | 12 | Helix arbustorum | 98.0† | |
| | 12 | Helix nemoralis | 101.3† | |
| | 10 | Helix hortensis | 101.3* | |
| | 12 | Limax maximus | 110.0 | |
| | 10 | Arion ater | 99.9† | |
| | 12 | Nereis virens | 102.7 | |
| | 12 | Planorbis corneus | 99.4 | |
| | 10 | Planorbis corneus | 103.2 | |
| | 12 | Sepia officinalis | 103.2 | |
| | 12 | Octopus vulgaris | 99.5 | |
| | 15 | Octopus vulgaris | 104.1 | |
| | 10 | Octopus vulgaris | 101.4 | |

TABLE X—*Continued*

TABLE X—*Continued*

| Origin of blood | Dilution of blood | Mixed with blood of | $s_{20}° \times 10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|
| | | | | *per cent* |
| Helix arbustorum L. | | | | |
| Upsala, Sweden | 5 | | 95.3* | |
| | 5 | | 94.3* | |
| | 12 | Helix pomatia | 96.3† | ±2 |
| Helix nemoralis (Müll.) | | | | |
| Upsala, Sweden | 8 | | 101.7* | |
| | 7 | | 100.2* | |
| | 6 | | 99.5* | |
| | 6 | | 95.2 | |
| | 12 | Helix pomatia | 100.8† | ±0 |
| Helix hortensis (Müll.) | | | | |
| Upsala, Sweden | 10 | | 99.2* | |
| | 10 | | 98.5* | |
| | 10 | Helix pomatia | 101.1* | ±0 |
| Limacidæ: | | | | |
| Agriolimax agrestis (L.) | | | | |
| Upsala, Sweden | 10 | | 95.9* | |
| | 10 | | 104.2 | |
| Limax maximus L. | | | | |
| Länna, Sweden | 12 | | 97.0† | |
| | 12 | Helix pomatia | 111.8† | ±2 |
| Arion ater L. | | | | |
| Länna, Sweden | 10 | | 93.7†(59.3) | |
| | 9 | | 110.5(63.2) | |
| | 9 | | 91.4(60.1) | |
| | 10 | Helix pomatia | 99.9(61.1)† | |
| | 10 | Lumbricus terrestris | (59.2)† | ±3 |
| Arion subfuscus Drap. | | | | |
| Länna, Sweden | 5 | | (64.3) | |
| | | Mean | 100.1 (16.2) | ±1.9 |
| | | | (61.9) | |
| | | | (133.6) | |

* Thickness of column of solution 0.8 cm.
† Solvent pH 5.
‡ Visible light, K3-filter, maximum of light absorption in the visible spectrum, 600 mμ.

*papillosa, Dendronotus arborescens* and *Patella vulgata* gave enough blood but did not show any of the absorption bands characteristic of the respiratory proteins.

According to some general remarks in the text-books of physiology (Winterstein, 1925), the blood of the lamellibranchians should, as a rule, contain hemocyanin. We have examined the following species, some of which are fairly large and easy to bleed, viz., *Unio pictorum, Anodonta cygnea, Dreissensia polymorpha, Cyprina islandica, Lævicardium norvegicum, Dosinia exoleta, Tellina crassa, Solen ensis, Solen siliqua, Mya arenaria, Ostrea edulis, Mytilus edulis, Volsella modiolus, Pecten opercularis.* In none of them were we able to detect the presence of any respiratory protein. The blood of the mussels has often a blueish opalescence accompanied by a slight light absorption which might have misled the earlier observers, but it is caused by the presence of coarse

TABLE XI

Conchifera: Lamellibranchiata

Pigment enclosed in corpuscles. Type of pigment, erythrocruorin. Solvent, 1 per cent NaCl; centrifugal force about 260,000 times gravity.

| Origin of blood | Dilution of blood | Thickness of column of solution | $s_{20°} \times 10^{13}$ |
|---|---|---|---|
| | | *cm.* | |
| Arcidæ: | | | |
| Arca pexata Say | | | |
| Woods Hole, Mass. | 5 | 0.2 | 3.40 |
| | 6 | 0.4 | 3.39 |
| | 6 | 0.4 | 4.09 |
| | 6 | 0.6 | 3.24* |
| | 20 | 1.2 | 3.20 |
| | | Mean | 3.46 |

* Solvent buffer pH 6.8; determined by Mrs. I.-B. Eriksson-Quensel.

suspended particles of unequal size settling rapidly when run in the ultracentrifuge and not by a blood pigment. A few lamellibranchians possess red corpuscles. We have had the opportunity to study only one species of this type, the blood-clam *Arca pexata.* All the other Mollusca have their respiratory protein dissolved in the blood plasma. Hemocyanin is the most common pigment but erythrocruorin occurs in a few species. Chlorocruorin and hemerythrin have not been found in this group. Tables VIII–XIII contain the determinations of sedimentation constants.

## TABLE XII

Conchifera: Cephalopoda: Decapoda

Pigment dissolved in plasma. Type of pigment, hemocyanin. Solvent, 1 per cent NaCl. Thickness of column of solution 1.2 cm. Standard species for mixture test, *Sepia officinalis* L. Centrifugal force about 75,000 times gravity.

| Origin of blood | Dilution of blood | Mixed with blood of | $s_{20}° \times 10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|
| | | | | *per cent* |
| Loliginidæ: | | | | |
| Loligo vulgaris Lam. | | | | |
| Naples, Italy..... | 10 | | 56.4 | |
| | 10 | Sepia officinalis | 55.1 | ±1 |
| | 15 | Sepia officinalis | 55.6 | ±1 |
| Sepiolidæ: | | | | |
| Sepiola oweniana d'Orb. | | | | |
| Kristineberg, Sweden | 12 | | 56.3 | |
| | 12 | | 56.4 | |
| | 20 | Sepia officinalis | 55.4‡ | ±0 |
| Rossia oweni Ball | | | | |
| Kristineberg, Sweden | 10 | | 54.6 | |
| | 15 | | 57.4 | |
| | 15 | Sepia officinalis | 55.7‡ | ±1 |
| | 12 | Octopus vulgaris | 55.1† | |
| Sepiidæ: | | | | |
| Sepia officinalis L. | | | | |
| Naples, Italy..... | 20 | | 54.8* | |
| | 25 | | 57.1 | |
| | 20 | | 55.9§ | |
| | 20 | | 56.5 | |
| | 20 | | 55.2† | |
| | 20 | Loligo vulgaris | 55.4 | |
| | 20 | Loligo vulgaris | 55.0 | |
| | 20 | Sepiola oweniana | 55.2‡ | |
| | 20 | Rossia oweni | 55.4‡ | |
| | 20 | Nereis virens | 60.4‖ | |
| | 20 | Lumbricus terrestris | 58.4 | |
| | 20 | Homarus vulgaris | 56.1 | |
| | 20 | Helix pomatia | 60.1‖ | |
| | 20 | Octopus vulgaris | 55.6 | |
| | | Mean | 56.2 | ±0.8 |

* Solvent pH 5.0.
† Solvent pH 6.8.
‡ Solvent pH 8.0.
§ Centrifugal force 115,000 times gravity.
‖ Centrifugal force 45,000 times gravity.

TABLE XIII

Conchifera: Cephalopoda: Octopoda

Pigment dissolved in plasma. Type of pigment, hemocyanin. Solvent, 1 per cent NaCl; thickness of column of solution 1.2 cm. Standard species for mixture test, *Octopus vulgaris* Lam. Centrifugal force 65,000 times gravity.

| Origin of blood | Dilution of blood | Mixed with blood of | $s_{20}$* × $10^{13}$ | Mixture test deviation from standard |
|---|---|---|---|---|
| Octopodidæ: | | | | *per cent* |
| Octopus vulgaris Lam. | | | | |
| Naples, Italy..... | 20 | | 50.6 | |
| | 20 | | 49.5 | |
| | 15 | | 48.4 | |
| | 15 | Rossia oweni | 48.7* | |
| | 20 | Sepia officinalis | 49.7 | |
| | 20 | Eledone cirrosa | 50.0 | |
| | 17 | Homarus vulgaris | 50.5 | |
| | 15 | Planorbis corneus | 51.0 | |
| | 20 | Helix pomatia | 53.4† | |
| | 15 | Helix pomatia | 54.5† | |
| Eledone moschata (Lam.) | | | | |
| Naples, Italy..... | 10 | | 48.9* | |
| Eledone cirrosa (Lam.) | | | | |
| Kristineberg, Sweden.. | 20 | | 46.4 | |
| | 20 | Octopus vulgaris | 49.8 | ±0 |
| | | Mean | 50.1 | |

* Solvent buffer pH 6.8.
† Centrifugal force 35,000 times gravity.

Six different sedimentation constants were found. The hemocyanin of the gastropods has the value $100.1 \times 10^{-13}$; the erythrocruorin of the same class $33.8 \times 10^{-13}$; the hemocyanin of the Amphineura, the Decapoda and the Octopoda 60.9, 56.2 and $50.1 \times 10^{-13}$ respectively, and the erythrocruorin of the lamellibranchians $3.46 \times 10^{-13}$. The constants 133.6, 61.9 and $16.2 \times 10^{-13}$ were observed for association and dissociation states of the gastropod hemocyanin. At the natural pH of the blood of the gastropods the dissociation product of constant 61.9, which is probably one-half the normal molecule, almost always appears. It is sometimes accompanied by the component of constant 16.2 (one-eighth of the normal molecule) and very seldom by the association product of constant 133.6. This latter molecule seems to be limited to the marine gastropods. It has been observed in the blood of *Littorina*, *Buccinum* and *Busycon*.

TABLE XIV

Eleutherozoa: Holothurioidea

Pigment enclosed in corpuscles. Type of pigment, erythrocruorin. Solvent, 1 per cent NaCl; thickness of column of solution 0.4 cm. Centrifugal force 250,000 times gravity.

| Origin of blood | Dilution of blood | $s_{20°} \times 10^{13}$ |
|---|---|---|
| Cucumariidæ: | | |
| Thyone briareus Les. | | |
| Woods Hole, Mass. | 6 | 2.60 |
| | 6 | 2.10 |
| | 6 | 2.95 |
| | 9 | 2.64 |
| | | Mean 2.57 |

*Echinoderma*

Only one representative of this group possessing respiratory blood pigment has been studied, viz., the sea cucumber, *Thyone briareus*. The protein, which is of the erythrocruorin type, is contained in blood corpuscles. The sedimentation constant is $2.57 \times 10^{-13}$. Table XIV gives the determinations.

*Tunicata*

No respiratory protein is recorded from these animals. We have examined the blood of *Ciona intestinalis* in the ultracentrifuge. It did not show any light absorption due to respiratory proteins.

TABLE XV

Cyclostomata: Hyperoartia, Hyperotreta

Pigment enclosed in corpuscles. Type of pigment, erythrocruorin. Solvent, 1 per cent NaCl + CO. Centrifugal force 225,000–305,000 times gravity.

| Origin of blood | Dilution of blood | Thickness of column of solution | $s_{20°} \times 10^{13}$ |
|---|---|---|---|
| | | cm. | |
| Petromyzontidæ: | | | |
| Lampetra fluviatilis (L.) | | | |
| Älvkarleby, Sweden | 9 | 0.4 | 1.98 |
| | 7 | 0.3 | 2.09 |
| Myxinidæ: | | | |
| Myxine glutinosa L. | | | |
| Kristineberg, Sweden | 6 | 0.2 | 2.33 |
| | 6 | 0.4 | 2.20 |
| | | | Mean 2.15 |

## TABLE XVI

Pisces: Selachii, Dipnoi, Teleostei

Pigment enclosed in corpuscles. Type of pigment, hemoglobin. Solvent, 1 per cent NaCl + CO. Centrifugal force 225,000–285,000 times gravity.

| Origin of blood | Dilution of blood | Thickness of column of solution | $s_{20°} \times 10^{13}$ |
|---|---|---|---|
| | | *cm.* | |
| Rajidæ: | | | |
| Raja clavata L. | | | |
| Kristineberg, Sweden | — | 0.6 | 4.21 |
| | — | 0.4 | 4.34 |
| Lepidosirenidæ: | | | |
| Protopterus annectens Owen | | | |
| Senegal | 2 | 0.3 | 4.52 |
| | 4 | 1.2 | 4.15 |
| Salmonidæ: | | | |
| Salmo irideus Gibb. | | | |
| Harbo fish-culture, Sweden | 4 | 0.4 | 4.14 |
| | 4 | 0.4 | 4.11 |
| Cyprinidæ: | | | |
| Cyprinus carassius | | | |
| Åkerlänna, Sweden | 5 | 0.4 | 4.38 |
| | 10 | 1.2 | 4.25 |
| | 6 | 0.3 | 4.63* |
| Anguillidæ: | | | |
| Anguilla anguilla (L.) | | | |
| Älvkarleby, Sweden | 10 | 0.4 | 4.21 |
| | 10 | 0.4 | 4.04 |
| Esocidæ: | | | |
| Esox lucius L. | | | |
| Upsala, Sweden | — | 1.2 | 4.40 |
| | — | 1.2 | 4.05 |
| Gasterosteidæ: | | | |
| Gasterosteus pungitius L. | | | |
| Upsala, Sweden | 10 | 1.2 | 4.45 |
| Percidæ: | | | |
| Lucioperca sandra Cuv. | | | |
| Upsala, Sweden | — | 1.2 | 4.48 |
| Tautoga onitis (L.) | | | |
| Woods Hole, Mass. | 10 | 0.4 | 4.12 |
| | 10 | 0.4 | 4.27 |

TABLE XVI—*Continued*

| Origin of blood | Dilution of blood | Thickness of column of solution | $s_{20}° \times 10^{13}$ |
|---|---|---|---|
| | | cm. | |
| Pleuronectidæ: | | | |
| Pleuronectes platessa L. | | | |
| Kristineberg, Sweden | — | 0.4 | 4.26 |
| Triglidæ: | | | |
| Prionotus carolinus (L.) | | | |
| Woods Hole, Mass. | 10 | 0.4 | 4.24 |
| | 10 | 0.4 | 4.49 |
| Blennidæ: | | | |
| Opsanus tau (L.) | | | |
| Woods Hole, Mass. | 10 | 0.4 | 4.21 |
| | 10 | 0.4 | 4.46 |
| | | | Mean 4.29 |

* Frozen for 41 days.

*Acrania*

A representative of this group, viz., *Branchiostoma lanceolatum,* was tried. We did not find it possible to draw enough blood from it for an ultracentrifugal test.

*Vertebrata*

The above measurements show that nowhere among the invertebrates has the respiratory protein hemoglobin characterized by the sedimentation constant $4.4 \times 10^{-13}$ and the active hemin group been met with. In view of this fact it became of great importance to study the red blood pigment of the various classes of the vertebrates in order to find out whether hemoglobin is the only respiratory blood protein of the higher animals. Tables XV–XX contain the determinations of the sedimentation constants.

The blood pigment from the lowest class of the vertebrates, the Cyclostomata, has a sedimentation constant of $2.15 \times 10^{-13}$ or less than half that of hemoglobin. Sedimentation equilibrium measurement by Mrs. I.-B. Eriksson-Quensel indicates that this pigment which we have classed among the erythrocruorins consists of a mixture of molecules of weight 17,500 and 34,500.

The mean values of the constants for Mammalia, Aves, and Pisces, viz., 4.29, 4.31, and $4.29 \times 10^{-13}$, are identical within the limits of error. The pigments from the reptiles and the amphibians very often show a second component of constant $7.14 \times 10^{-13}$ probably corresponding to a double molecule. This association product seems to increase

## TABLE XVII

**Amphibia: Urodela, Anura**

Pigment enclosed in corpuscles. Type of pigment, hemoglobin. Solvent, 1 per cent NaCl + CO; centrifugal force 195,000–290,000 times gravity.

| Origin of blood | Dilution of blood | Thickness of column of solution | $s_{20°} \times 10^{13}$ |
|---|---|---|---|
| | | cm. | |
| Salamandridæ: | | | |
| Salamandra maculosa Laur. | | | |
| Germany | — | 1.2 | 4.89(7.66) |
| | — | 1.2 | 4.96(6.24) |
| | 10 | 0.6 | 4.60* |
| | — | 0.6 | 4.83* |
| Bufonidæ: | | | |
| Bufo viridis Laur. | | | |
| Germany | — | 1.2 | 4.72(7.54) |
| | — | 1.2 | 4.83(7.27) |
| Bufo valliceps Wiegm. | | | |
| Honduras | — | 0.6 | 4.82† |
| | — | 0.6 | 4.73*(7.73, 12.53) |
| Ranidæ: | | | |
| Rana temporaria L. | | | |
| Upsala, Sweden | — | 1.2 | 4.28 |
| | — | 1.2 | 4.62 |
| | | | 4.73(7.29) Mean |

* Centrifugal force 365,000 times gravity.
† Second computation incalculable.

on standing. Sometimes a third component about $12 \times 10^{-13}$ (probably a triple molecule) was observed in old samples of reptile blood. The constant of the normal component is $4.71 \times 10^{-13}$ or slightly higher than that for the mammals, birds, and fishes.

The difference is believed to be real. Tests carried out at high speed (72,000 r.p.m. corresponding to a centrifugal force 380,000 times gravity) have not given any indication of a composite character of the 4.7 component. Sedimentation equilibrium measurements will be required to decide whether this deviation is due to a real difference in molecular weight or merely to a slightly different shape of the hemoglobin molecule of the reptiles and amphibians. Some recent determinations of sedimentation constants and molecular weights carried out by K. O. Pedersen on horse blood hemoglobin have shown that this molecule tends to dissociation at high dilutions. We have observed such a phenomenon even more strikingly in one case of cat's blood hemoglobin. Here dissociation into half molecules took place even at moderate dilutions.

## TABLE XVIII

**Reptilia: Testudinata, Squamata**

Pigment enclosed in corpuscles. Type of pigment, hemoglobin. Solvent, 1 per cent NaCl + CO; centrifugal force 220,000–290,000 times gravity.

| Origin of blood | Dilution of blood | Thickness of column of solution | $s_{20}° \times 10^{13}$ |
|---|---|---|---|
| | | cm. | |
| Testudinidæ: | | | |
| Chrysemys picta (Schneid.) | | | |
| N. America | — | 0.6 | 4.47(6.97) |
| | — | 0.6 | 4.38(6.67) |
| Anguidæ: | | | |
| Anguis fragilis L. | | | |
| Germany | — | 0.3 | 4.96 |
| | 30 | 0.6 | 4.47* |
| | 30 | 0.6 | 5.00* |
| Lacertidæ: | | | |
| Lacerta vivipara Jacq. | | | |
| Germany | — | 0.6 | 4.20(6.96) |
| | — | 0.6 | 4.90(7.07) |
| | 10 | 0.6 | 4.67(7.30) |
| | 20 | 0.6 | 4.50† |
| Chamæleontidæ: | | | |
| Chamæleon chamæleon (L.) | | | |
| S. Europe | — | 0.4 | 4.57 |
| Colubridæ: | | | |
| Coluber longissimus (Laur.) | | | |
| S. Europe | 5 | 0.3 | 4.67 |
| | 5 | 0.3 | 4.75 |
| | 10 | 0.6 | 5.04 |
| | 20 | 0.6 | 4.72 |
| | 20 | 0.6 | 4.80 |
| | 20 | 0.6 | 4.66* |
| | 60 | 2.2 | 4.98 |
| | | | Mean 4.69(6.99) |

* Centrifugal force 380,000 times gravity.
† Second computation not calculable.

### SEDIMENTATION RATIOS

In order to check the above determinations and bind together the higher constants into a system of relative values, a number of ultracentrifugal runs were carried out on mixtures containing two blood pigments of different sedimentation constants. Such relative measurements are possible only in the case of rapid sedimentation where the

TABLE XIX

Aves: Gallinacei, Columbæ, Lamellirostres, Striges, Pici, Passeres

Pigment enclosed in corpuscles. Type of pigment, hemoglobin. Solvent, 1 per cent NaCl + CO. Centrifugal force, 230,000–380,000 times gravity.

| Origin of blood | Dilution of blood | Thickness of column of solution | $s_{20}° \times 10^{13}$ |
|---|---|---|---|
| | | cm. | |
| Phasianidæ: | | | |
| Gallus gallus L. | | | |
| Upsala, Sweden | 30 | 0.3 | 4.13 |
| | 30 | 0.3 | 4.31 |
| Columbidæ: | | | |
| Columba livia L. v. domestica | | | |
| Upsala, Sweden | — | 1.2 | 4.46 |
| | — | 1.2 | 4.33 |
| Anatidæ: | | | |
| Anas platyrhyncha L. v. domestica | | | |
| Upsala, Sweden | 40 | 0.6 | 4.41 |
| | 40 | 1.2 | 4.34 |
| Strigidæ: | | | |
| Syrnium aluco L. | | | |
| Upsala, Sweden | — | 0.6 | 4.28 |
| | — | 0.6 | 4.39 |
| Picidæ: | | | |
| Picus viridis L. | | | |
| Upsala, Sweden | 40 | 0.4 | 4.37 |
| | 80 | 1.2 | 4.23 |
| Paridæ: | | | |
| Parus major L. | | | |
| Upsala, Sweden | 30 | 1.2 | 4.35 |
| | 15 | 0.6 | 4.21 |
| Corvidæ: | | | |
| Corvus cornix L. | | | |
| Upsala, Sweden | 20 | 0.6 | 4.45 |
| | 44 | 1.2 | 4.25 |
| | | | Mean 4.32 |

diffusion is low and the boundaries therefore fairly sharp. This condition is not fulfilled for the pigments contained in corpuscles. The individual values obtained from these runs have already been used. In Table XXI we have recorded the observed sedimentation ratios as well as the ratios calculated from the mean values of the different types of the sedimentation constant.

## TABLE XX

Mammalia: Insectivora, Rodentia, Carnivora, Ungulata, Primates

Pigment enclosed in corpuscles. Type of pigment, hemoglobin. Solvent, 1 per cent NaCl + CO. Centrifugal force 210,000–330,000 times gravity.

| Origin of blood | Dilution of blood | Thickness of column of solution | $s_{20°} \times 10^{13}$ |
|---|---|---|---|
| | | *cm.* | |
| Erinaceidæ: | | | |
| Erinaceus europæus L. | | | |
| Upsala, Sweden | 20 | 0.3 | 4.42 |
| | 20 | 0.3 | 4.57 |
| Leporidæ: | | | |
| Oryctolagus cuniculus L. | | | |
| Upsala, Sweden | 20 | 0.3 | 4.27 |
| | 20 | 0.3 | 4.44 |
| Felidæ: | | | |
| Felis domestica Schreber | | | |
| Upsala, Sweden | 30 | 0.3 | 4.26 |
| | 30 | 0.3 | 4.17 |
| Canidæ: | | | |
| Canis familiaris L. | | | |
| Upsala, Sweden | 25 | 0.3 | 4.29 |
| | 60 | 0.6 | 4.11 |
| Equidæ: | | | |
| Equus caballus L. | | | |
| Upsala, Sweden | | 0.6 | 4.40 |
| | | | 4.42* |
| Bovidæ: | | | |
| Bos taurus L. | | | |
| Upsala, Sweden | 30 | 0.3 | 4.18 |
| | 30 | 0.3 | 4.36 |
| Cercopithecidæ: | | | |
| Cercopithecus sp. | | | |
| W. Africa | 40 | 0.6 | 3.94 |
| | 20 | 0.3 | 3.86 |
| | 27 | 0.3 | 4.22 |
| Hominidæ: | | | |
| Homo sapiens | | | |
| | — | 0.6 | 4.54 |
| | 20 | 0.3 | 4.42 |
| | | | Mean 4.29 |

* Mean of four determinations by K. O. Pedersen.

The observed and calculated ratios agree within the limits of experimental error. From the observed ratios and the sum of all the absolute values of sedimentation constants a set of relative values may be calculated. In Table XXII these constants together with the absolute values found by us and those determined by other investigators are given.

TABLE XXI

Observed and calculated sedimentation ratios for respiratory pigments dissolved in the blood of the invertebrates, and showing two boundaries when subjected to the mixture test.

| Species | Sedimentation Ratio | | Deviation from calc. ratio |
|---|---|---|---|
| | Observed | Calculated | |
| | | | *per cent* |
| Helix pomatia/Nereis virens | 1.72 | 1.75 | −1.7 |
| Helix pomatia/Sepia officinalis | 1.72 | 1.75 | −1.7 |
| Helix pomatia/Octopus vulgaris | 1.86 | 1.99 | −7.0 |
| Helix pomatia/Planorbis corneus | 2.92 | 2.95 | −1.0 |
| Lumbricus terrestris/Nereis virens | 1.08 | 1.08 | ±0 |
| Lumbricus terrestris/Arenicola marina | 1.07* | 1.08 | −0.9 |
| Nereis virens/Planorbis corneus | 1.67 | 1.68 | −0.6 |
| Sepia officinalis/Octopus vulgaris | 1.12 | 1.14 | −1.8 |
| Sepia officinalis/Homarus vulgaris | 2.44 | 2.43 | +0.4 |
| Rossia oweni/Octopus vulgaris | 1.13 | 1.14 | −0.9 |
| Octopus vulgaris/Planorbis corneus | 1.51 | 1.48 | +2.0 |
| Octopus vulgaris/Homarus vulgaris | 2.29 | 2.13 | +7.5 |
| Planorbis corneus/Homarus vulgaris | 1.33 | 1.44 | −8.3 |
| Calocaris macandreæ/Homarus vulgaris | 1.50 | 1.44 | +4.2 |
| Homarus vulgaris/Pandalus borealis | 1.41 | 1.39 | +1.4 |
| Homarus vulgaris/Palæmon fabrici | 1.39 | 1.39 | ±0 |
| Homarus vulgaris/Eupagurus bernhardus | 1.29 | 1.39 | −7.8 |
| | | | mean dev. −0.95 |

* Determined by Inga-Britta Eriksson-Quensel.

## DISCUSSION OF RESULTS

One of the most striking points borne out by the above investigation is, perhaps, the fact that low sedimentation constants were always found for the pigments enclosed in red blood corpuscles. Only four different values were observed, viz., 2.1, 2.6, 3.5 and $4.4 \times 10^{-13}$, probably corresponding to molecules of mass $1/2 \times 34{,}500$, $1 \times 34{,}500$, a mixture of

TABLE XXII

Absolute and relative values of the seven highest sedimentation constants of blood pigments.

| Standard species | $s_{20°} \times 10^{13}$ | | | | |
|---|---|---|---|---|---|
| | Mean absolute value | | | | Relative value |
| | Present invest. | No. determinations | Other invest. | No. determinations | |
| Helix pomatia | 100.1 | 58 | 100.1 | 39* | 98.3 |
| Lumbricus terrestris | 61.7 | 13 | 60.9 | 14† | 61.8 |
| Nereis virens | 57.0 | 61 | 57.4 | 15† | 57.1 |
| Octopus vulgaris | 50.1 | 13 | 51.7 | 29† | 51.4 |
| Planorbis corneus | 33.9 | 25 | 33.7 | 11† | 33.9 |
| Homarus vulgaris | 23.5 | 35 | 23.1 | 11† | 23.6 |
| Pandalus borealis | 16.9 | 19 | 16.1 | 18† | 17.1 |

* Determinations by Inga-Britta Eriksson-Quensel and K. O. Pedersen.
† Determinations by Inga-Britta Eriksson-Quensel.

both, and $2 \times 34{,}500$ containing 1, 2, and 4 atoms of iron respectively. The first three values are represented in the invertebrates and in the lowest class of the vertebrates, the Cyclostomata, while the last value is found exclusively in the higher classes of the vertebrates. Among the amphibians and reptiles a higher component of constant $7.1 \times 10^{-13}$, probably corresponding to a molecule built up of two normal hemoglobin molecules, was sometimes observed. The reddish-brown corpuscles found in the cœlomic fluid of the sipunculoid worms contain an entirely different protein, the hemerythrin, which has, however, a sedimentation constant of the same order of magnitude as the hemoglobin of the higher vertebrates. From the standpoint of blood kinship it would seem reasonable to assume that the vertebrates have developed from some invertebrate group possessing blood corpuscles and characterized by a red respiratory pigment of the same sedimentation constant and molecular weight. As a matter of fact, the lowest class of the vertebrates, the Cyclostomata, possesses a respiratory blood protein which with regard to sedimentation constant resembles closely the pigment contained in the erythrocytes of the capitellide worms. It is of interest to note that H. E. Jordan (1933) arrives at a similar conclusion from the consideration of histological data.

The blood pigments which occur dissolved in the plasma are as a rule characterized by high sedimentation constants and consequently by high molecular weights. The only exception so far observed is the red pigment of the *Chironomus* larvæ, which has a constant identical with that

of the red pigment contained in the erythrocytes of the capitellide worms. It might seem tempting to put forward the hypothesis that the giant pigment molecules dissolved in the blood plasma play, to a certain extent at least, the rôle of blood corpuscles. On the other hand, it must be borne in mind that even the largest of these molecules, that of the Gastropoda hemocyanin, has a diameter of only 1/200 of that of a red blood corpuscle.

The remarkable constancy of the mass and shape properties of the molecules of the respiratory proteins within closed animal groups, as expressed by the identity of the sedimentation constants, is probably a measure of the similarity of the chemical processes which lead to the formation of these pigments. To a certain extent, therefore, identity of sedimentation constant may be taken as a criterion of biological kinship. From this point of view it is of interest to note that the hemocyanins of the very old arthropod genera *Limulus* and *Euscorpius* have the same sedimentation constant, viz., $34 \times 10^{-13}$. Only two other genera, the crustacean *Calocaris*, the blood of which contains hemocyanin, and the red-blooded snail *Planorbis* have the same constant.

One must, however, be very careful when trying to trace relationships between the various classes of animals from the properties of their blood pigments. The above investigation has shown that the number of different sedimentation constants is very restricted (so far only 14 have been observed) and that the same constant occurs in respiratory proteins containing different active groups. It is therefore not unprobable that the same constant may sometimes be represented in animal classes which could hardly be looked upon as nearly related. As an example of this kind, one might mention the erythrocruorin of *Lumbricus* and the hemocyanin of *Tonicella*, which have the same sedimentation constant, viz., $61 \times 10^{-13}$.

The observations about the behaviour of the blood pigments near the borders of the pH stability regions as well as the systematic study of the entire stability ranges and the determination of their molecular weights from sedimentation equilibrium measurements of some of these proteins, now being carried out by Mrs. I.-B. Eriksson-Quensel, seem to indicate that the blood protein molecules of high sedimentation constants break up into definite units at certain critical pH values and that these dissociation products are identical with the blood proteins of lower molecular weight as far as the mass and shape properties go. It seems, therefore, that only a few molecular masses are stable and that it would depend upon the composition of the molecule with regard to the various amino acids or other constituents and upon the actual pH of the solution whether one or the other of the different possibilities is realized.

A detailed investigation of the isoelectric points of the respiratory proteins by Dr. K. O. Pedersen (1933) of this laboratory has shown, however, that, as a rule, each species is characterized by a special value of the isoelectric point of its blood pigment. The chemical composition of the respiratory protein therefore varies from one species to the other even in cases where the sedimentation constants are identical. No relationship has so far been found between the position of the isoelectric point and the mass of the molecule. At the present time we are not able to predict what sedimentation constant a respiratory protein of known chemical composition would have, but it is believed that this will become possible with increasing knowledge of the constitution of the protein molecule.

The investigation has been made possible by financial help from the Rockefeller Foundation, the Nobel Foundation, the Foundation Therese och Johan Anderssons Minne and from the Astra Co. Most of the marine species were put at our disposal by the Zoölogical Station at Kristineberg, Sweden. We wish to express our sincere thanks to the Prefect, Professor E. Lönnberg, for his kindness in allowing these collections to be made for us. To the Director of the Station, Dr. G. Gustafson, we are greatly indebted for his constant readiness to provide us with the desired species, and for the care which he has taken in finding and collecting them for us. A number of samples of blood from marine animals were kindly sent us from the Zoölogical Station at Naples. We are most grateful to Professor R. Dohrn and Dr. L. Califano for this courtesy. We likewise wish to extend our hearty thanks to Dr. A. C. Redfield, of the Oceanographic Institution at Woods Hole, Mass., for his kindness in helping us to procure samples of blood from some especially interesting marine species not found in Europe. Professor S. Ekman, Dr. I. Arwidsson of the Department of Zoölogy, Upsala, and Professor N. Holmgren of the Department of Zoölogy, Stockholm, have given us valuable help with regard to land and freshwater animals.

## Summary

1. A systematic study of the sedimentation constants of the respiratory blood proteins throughout the animal kingdom has been carried out by means of the ultracentrifugal method.

2. Respiratory proteins enclosed in corpuscles have low sedimentation constants. The following values were found for erythrocruorin: capitellide worms and Cyclostomata, $2.1 \times 10^{-13}$; glyceride worms and lamellibranchians, $3.5 \times 10^{-13}$; holothurians, $2.6 \times 10^{-13}$. Hemoglobin characterized by the sedimentation constant $4.4 \times 10^{-13}$ occurs only in

the five higher classes of the vertebrates, viz., Mammalia, Aves, Reptilia, Amphibia, Pisces. Among the reptiles and amphibians an association product of hemoglobin with sedimentation constant $7.1 \times 10^{-13}$, probably representing a doublet, was often found. This product was never observed in the other classes of the vertebrates.

3. Respiratory proteins dissolved in the plasma have, as a rule, high sedimentation constants. The only exception is the erythrocruorin of the *Chironomus* larvæ, which has the constant $2.0 \times 10^{-13}$. All polychæte worms and hirudineans with dissolved pigment have the constant $57.5 \times 10^{-13}$ (erythrocruorin and chlorocruorin). A dissociation product of constant $11.7 \times 10^{-13}$ (probably 1/16 of the normal molecule) is sometimes found. The oligochæte worms have a variety of erythrocruorin of slightly higher constant, $61.9 \times 10^{-13}$. The crustaceans show as a rule two sedimentation constants, 16.9 and $23.5 \times 10^{-13}$. The former one, which occurs both in erythrocruorins and hemocyanins, probably represents a molecular weight of one-half of the latter. Species characterized by the latter constant have hemocyanin and give a mixture of both constants in alkaline solution, thus demonstrating the dissociation into half molecules. One crustacean species (*Calocaris macandreae*) has a hemocyanin constant of $34.0 \times 10^{-13}$. This sedimentation constant is also characteristic of the hemocyanin of the scorpion (*Euscorpius carpaticus*). The xiphosuran (*Limulus polyphemus*) has a hemocyanin of the same constant, but its blood contains two more hemocyanin varieties of constants 16.5 and $59.1 \times 10^{-13}$ and a dissociation product of constant $6 \times 10^{-13}$. The erythrocruorin of the gastropods has the same constant, $33.8 \times 10^{-13}$. The normal hemocyanin of the gastropods, on the other hand, is characterized by the constant $100.1 \times 10^{-13}$. It forms an association product of constant $133.6 \times 10^{-13}$ and two dissociation products of constants 16.2 and $61.9 \times 10^{-13}$ (probably 1/2 and 1/8 of the normal molecule respectively). The constant $60.9 \times 10^{-13}$ is also found in the amphineuran hemocyanin (*Tonicella marmorea*). The cephalopods show two constants, $56.2 \times 10^{-13}$ for the decapods and $50.1 \times 10^{-13}$ for the octopods. The latter constant has not been observed in any other place and is the only sedimentation exclusively characteristic of an animal group.

4. The significance of the data collected in the present investigation cannot be fully understood until a sufficient number of pH-stability curves and sedimentation equilibrium measurements have been made on respiratory proteins. The few determinations of this kind so far available, however, seem to indicate that all native proteins form a closed system in which only a very limited number of mass and shape types

are stable. Reversible association and dissociation reactions take place easily when the pH of the solution is slightly changed. Within a well-defined animal group all species have as a rule respiratory pigments of the same sedimentation constant (or constants) and dissociate in a similar way. Biological kinship, therefore, is usually accompanied by identity in the sedimentation constants. On the other hand, owing to the small number of different constants possible, the same constant must of necessity occur in different animal groups.

## REFERENCES

JORDAN, H. E., 1933. *Quart. Rev. Biol.*, **8**: 58.
PEDERSEN, K. O., 1933. *Kolloid-Zeitschr.*, **63**: 268.
REDFIELD, A. C., 1933. *Quart. Rev. Biol.*, **8**: 31.
SVEDBERG, T., 1933. *Jour. Biol. Chem.*, **103**: 311.
SVEDBERG, T., AND I.-B. ERIKSSON-QUENSEL, 1933. *Jour. Am. Chem. Soc.*, **55**: 2834.
SVEDBERG, T., AND A. HEDENIUS, 1933. *Nature*, **131**: 325.
SVEDBERG, T., 1934. *Chem. Rev.*, **14**: 1.
WINTERSTEIN, H., 1925, *Handb. d. vergl. Physiol.*, 1(1): 652.

# THE MALE SOMATIC CHROMOSOME GROUP IN SCIARA PAUCISETA [1]

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## INTRODUCTION

The present study is designed to furnish additional evidence bearing on the general problem of sex determination in *Sciara*. It is concerned particularly with two features. In the first place, evidence is given showing that in *Sciara pauciseta* Felt there are seven chromosomes in the male soma instead of eight as in the female soma, although the germ-line chromosome groups are alike in the two sexes. In the second place, it is shown that this evidence adds support to the hypothesis that the "precocious" [2] chromosome in *Sciara* is a sex chromosome (Metz, 1929, 1930).

Earlier evidence bearing on the immediate problem may be summarized briefly as follows:

1. In all species of *Sciara* thus far adequately studied, the chromosome group of the germ-line in both sexes (*i.e.*, spermatogonia and oögonia) consists of symmetrical pairs of chromosomes—except for variations in the number of "limited" chromosomes which are found only in the germ-line and hence do not concern us at present.[3]

2. The chromosome group of the female soma in these species is regularly composed of four symmetrical pairs.

3. The chromosome group of the male soma in *Sciara coprophila* is composed of three symmetrical pairs of chromosomes and an unpaired chromosome. The eighth chromosome found in the female soma is eliminated during cleavage in the somatic line of the male. (Metz, 1931*a*; Du Bois, 1932.)

4. Genetic evidence indicates that this unpaired chromosome is regularly the maternal X-chromosome, and that, therefore, it is the paternal

[1] This investigation has been aided by a grant from the National Research Council, Committee for Research in Problems of Sex; grant administered by C. W. Metz.

[2] For explanation of "precocious" chromosome, see No. 6 under the "earlier evidence" in the Introduction.

[3] See Metz, Moses, and Hoppe, 1926; Metz, 1926, 1931*a* and *b*; Metz and Schmuck, 1931*b*.

sex chromosome which is regularly eliminated from the male soma. (Metz, *loc. cit.*; Metz and Schmuck, 1931*a*.)

5. Available evidence makes it seem probable that this paternal sex chromosome is an X, not a Y, and that sex in the male is determined by the constitution of the soma. It has not been possible, however, to demonstrate this.

6. In all species of *Sciara* in which spermatogenesis has been studied, the second spermatocyte division exhibits peculiarities resulting in the transmission to all sperms of both halves of one chromosome called the "precocious" chromosome. It has been suggested that this chromosome is a sex chromosome and that its behavior may have a bearing on sex determination (Metz, 1930, 1931*b*).

7. So far as known, all species of *Sciara* possess four pairs of chromosomes in the female somatic cells. The groups are not all alike, however, in respect to shape and size of the chromosomes. Some species possess three pairs of rod-like and one pair of V-shaped chromosomes, while in others there are two pairs of rods and two pairs of V's. In species of the first group the "precocious" chromosome is regularly one of the rod-like members, and in those of the second it is regularly one of the V-shaped members (Metz, Moses, and Hoppe, *loc cit.*; and unpublished evidence of C. W. Metz).

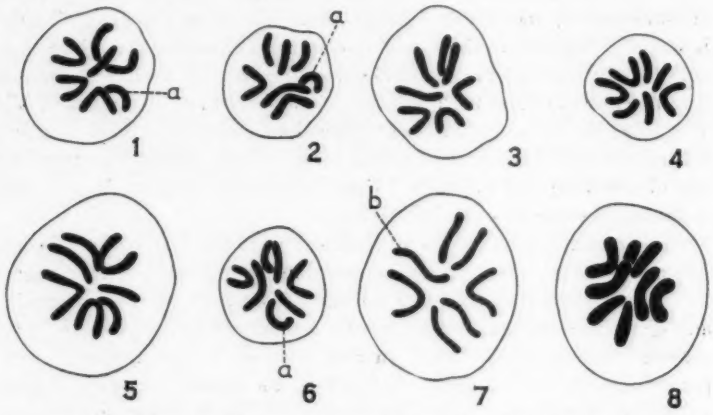
It is evident from the above résumé that if the "precocious" chromosome is a sex chromosome as postulated, it should regularly agree in size and form with the one eliminated from the male somatic nuclei, and hence the examination of species having different types of "precocious" chromosomes should furnish evidence for or against the hypothesis under consideration. Of course, the method can give support to the hypothesis only in an indirect manner, but it might provide conclusive contrary evidence if the hypothesis is incorrect. In the case of *Sciara coprophila,* already noted, there is agreement between the "precocious" chromosome and the sex chromosome, both of which are rod-like. The present study deals with a representative of the other type and shows that there is also an agreement where the "precocious" chromosome is V-shaped.

## Observations

The present observations were made on young larvæ. The specimens were dissected, fixed in Flemmings' fluid (strong), and stained by the Feulgen method. This stain gives excellent results, since only the chromatin becomes colored. The migratory cells of the nervous system, as well as other somatic cells, were used. The former are particularly good because, although few in number, they are large and the mitotic figures are clear, with the chromosomes sharply defined.

The female somatic group in this species consists of two pairs of rod-shaped and two pairs of V-shaped chromosomes. An example is shown in Fig. 8. (See also Metz, 1926, Fig. 34.) The shapes are, of course, determined by the point of spindle fiber attachment, which is terminal in the rod-like and median in the V-shaped members. A further distinction is seen in the size of the two types—the V-shaped chromosomes being considerably longer than the rod-like ones in most cases.

The male somatic groups, obtained from a large number of individuals, all show seven chromosomes—two pairs of rods, one pair of V's and an unpaired V. Seven such groups are shown in Figs. 1 to 7, from camera lucida drawings made from sectioned material. Frequently a drawing does not show accurately the shape of the chromo-




FIGS. 1–8. Somatic chromosome groups of *Sciara pauciseta* Felt. Figures 1–7 from male somatic cells. Figure 8 from somatic cell of female (ovarian follicle cell). Drawings for FIGS. 1–7 made by Miss Louise H. Buck.

somes, since they may not be flat on the metaphase plate. Figures 3, 4, and 5 show most clearly the three V's and four rods. Careful detailed studies of the chromosome marked "*a*" in Figs. 1, 2 and 6 show that it is a rod and not a V as might be thought at first glance. In Fig. 7 the chromosome marked "*b*" is a V, although in the drawing it appears nearly straight.

Although the actual process of chromosome elimination during cleavage has not been observed in the present species, it seems clear from the evidence presented above, together with that cited from earlier studies on spermatogonia and female somatic chromosome groups, that a V-shaped sex chromosome is eliminated from male somatic cells here

just as a rod-like one is eliminated in *Sciara coprophila*. Since in the present species the "precocious" chromosome is V-shaped, it is clear that the evidence, so far as it goes, supports the hypothesis that the "precocious" chromosome is a sex chromosome.

The author is deeply indebted to Dr. C. W. Metz for his valuable help in working out this problem.

## LITERATURE

METZ, C. W., 1926. Chromosome Studies on Sciara (Diptera). I. Differences between the chromosomes of the two sexes. *Am. Nat.*, **60**: 42.

METZ, C. W., MILDRED S. MOSES, AND ELLA N. HOPPE, 1926. Chromosome Behavior and Genetics in Sciara (Diptera). I. Chromosome behavior in the spermatocyte divisions. *Zeitschr. f. induk. Abs. u. Vererb.*, **42**: 237.

METZ, C. W., 1929. Evidence that "Unisexual" Progenies in Sciara are due to Selective Elimination of Gametes (Sperms). *Am. Nat.*, **63**: 214.

METZ, C. W., 1929. Sex Determination in Sciara. *Am. Nat.*, **63**: 487.

METZ, C. W., 1930. A Possible Alternative to the Hypothesis of Selective Fertilization in Sciara. *Am. Nat.*, **64**: 380.

METZ, C. W., 1931*a*. Chromosomal Differences between Germ cells and Soma in Sciara. *Biol. Zentrlb.*, **51**: 119.

METZ, C. W., 1931*b*. Unisexual Progenies and Sex Determination in Sciara. *Quart. Rev. Biol.*, **6**: 306.

METZ, C. W., AND M. L. SCHMUCK, 1931*a*. Studies on Sex Determination and the Sex Chromosome Mechanism in Sciara. *Genetics*, **16**: 225.

METZ, C. W., AND M. LOUISE SCHMUCK, 1931*b*. Differences between Chromosome Groups of Soma and Germ-line in Sciara. *Nat. Acad. Sci.*, **17**: 272.

DU BOIS, ANNE MARIE, 1932. Elimination of Chromosomes during Cleavage in the Eggs of Sciara (Diptera). *Nat. Acad. Sci.*, **18**: 352.

# EFFECTS OF CENTRIFUGAL FORCE ON THE ECTOPLASMIC LAYER AND NUCLEI OF FERTILIZED SEA URCHIN EGGS

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In previous studies (E. B. Harvey, 1932, 1933*a*, 1933*b*) of sea urchin eggs stretched and broken apart by centrifugal force, it was found that unfertilized and fertilized eggs behave quite differently. The former form dumb-bells and pull apart as spheres of very definite size, while the latter during a certain period (monaster stage) form long streamers which break irregularly. A more extensive study has now been made of the fertilized eggs of some of the European sea urchins in which, owing to the clear, unpigmented protoplasm, the nuclear phenomena can be seen with great clearness in the living egg. It is possible by centrifugal force to separate the male and female pronuclei so that they come to lie either in different regions of an elongated egg, or in completely separated fragments. A study of the development of these eggs and fragments is presented in this paper, together with observations on the outermost layer of the fertilized egg (the ectoplasmic layer), which is actually peeled off the egg by centrifugal force. It has also been found that if eggs are centrifuged just before cleavage, the cleavage plane may come in without any relation to the new position of the mitotic figure.

## Ectoplasmic Layer

The ectoplasmic or hyaline plasma layer of the sea urchin egg is a thin colorless layer appearing on the surface of the egg following fertilization, and is quite distinct from the fertilization membrane. It has an important function in holding the blastomeres together; the layer is disorganized and the blastomeres fall apart in the absence of calcium (Herbst, 1900; Gray, 1924, 1931; Moore, 1930). Some investigators (Ziegler, 1903; Gray, 1924, 1931; and Just, 1928) have considered it of importance in the mechanics of cell division, albeit for different rea-

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sons. The characteristic heaping of the layer at the equator at the beginning of cleavage would certainly lead one to believe that it is in some way concerned with the cleavage process.

In most sea urchins, the Naples species, *Paracentrotus lividus, Parechinus microtuberculatus, Sphærechinus granularis,* and the Woods Hole species, *Arbacia punctulata,* the layer, though detectable, is extremely thin (less than 1 $\mu$) until ten or fifteen minutes after fertilization and it gradually becomes thicker until it measures 2 or 3 $\mu$ before cleavage (Fig. 1). In the Naples species of *Arbacia, A. pustulosa,* the ectoplasmic layer is well formed at the time that the fertilization membrane lifts off and even then measures 2–3 $\mu$. It is particularly striking in this species as it makes a sharp contrast with the deeply colored egg. It is perfectly apparent here, by measuring the same egg (with a water immersion lens) before and after insemination, that the ectoplasmic layer is *added on* to the surface of the unfertilized egg. The diameter of the total egg increases 4–5 $\mu$; this increase cannot be caused by any flattening of the egg due to gravity since the diameter of the colored portion remains the same. (See E. N. Harvey, 1933.) Glaser (1914, 1924) found (also by direct measurement) a *decrease* in diameter of the *Arbacia punctulata* egg on fertilization. In this species, however, it is to be noted that the ectoplasmic layer is extremely thin at this time so that any change in diameter due to it could scarcely be measured. His results have been questioned by Chambers (1921) and others. The layer seems to be, morphologically, in the nature of an extracellular membrane, rather than an integral part of the egg protoplasm, though this in no way detracts from its physiological importance. The behavior of the layer when subjected to centrifugal force leads to the same conclusion.

When the eggs of any of these sea urchins are centrifuged at any time after ten minutes following insemination, in a sucrose solution of the same density and tonicity as the eggs (in order to keep them suspended), at a centrifugal force of about 5000 $\times$ g, the ectoplasmic layer is centrifuged off the egg, usually in the form of a ring. It is best observed in eggs which have a large perivitelline space such as *Parechinus* (20 $\mu$) and *Paracentrotus* (12 $\mu$). When the eggs are first removed from the centrifuge, the ring is seen encircling the slightly tapering heavy pole of the egg, and the characteristic ectoplasmic layer investing the surface of the normal fertilized egg is lacking (Fig. 2, Photograph 1). As the egg becomes spherical, the ring slips off and lies free in the perivitelline space (Fig. 3). It remains here through cleavage and can be seen attached to the blastula (Fig. 4) even after it has become free-swimming, but eventually drops off. The ring is not always perfect, but is often incomplete or broken or has vacuolated or thinner

areas (Fig. 5). If eggs are centrifuged 7–10 minutes after fertilization, the ring is very narrow; if centrifuged before that, the ring does not form since there is an insufficient amount of ectoplasmic material. It may be centrifuged off also in the 2- and 4-cell stages, but is then of smaller size and more irregular shape (Fig. 6). The ring (and also the intact ectoplasmic layer) does not stain with any intravitam dyes; in such dyes as methylene blue, brilliant cresyl blue, and neutral red, it remains quite colorless, forming a sharp contrast to the heavily staining granular protoplasm. The ectoplasmic layer of the *Arbacia* egg (both *A. punctulata* and *A. pustulosa*) can also be thrown off, but owing to the small perivitelline space (1–5 $\mu$), it is usually difficult to observe unless the fertilization membrane is broken at the centripetal pole and has slipped partly off, leaving a large space between it and the surface of the egg at the centrifugal pole. (Photograph 2.) In *Arbacia punctulata* it usually appears as a crescent rather than a ring. After the ectoplasmic layer from any of the eggs with intact fertilization membranes has been centrifuged off, it is gradually reformed on the surface of the egg, so that in about thirty minutes it looks almost the same as in uncentrifuged eggs, and after cleavage the blastomeres cohere. Perfectly normal plutei develop from these eggs in as large a percentage as from the controls. The ectoplasmic layer must also be replaced during the development of isolated blastomeres separated in calcium-free sea water, since, when returned to sea water, they may form perfect dwarf plutei whose cells cohere. It will be noted that the ectoplasmic layer differs markedly in this respect from the fertilization membrane, which is formed only once and is never replaced when once removed.

The ring-formation from the ectoplasmic layer, as described above, takes place in eggs centrifuged in an isotonic sucrose solution brought to the same density as the eggs by the addition of sea water; after centrifuging, the eggs were immediately returned to sea water. If the eggs

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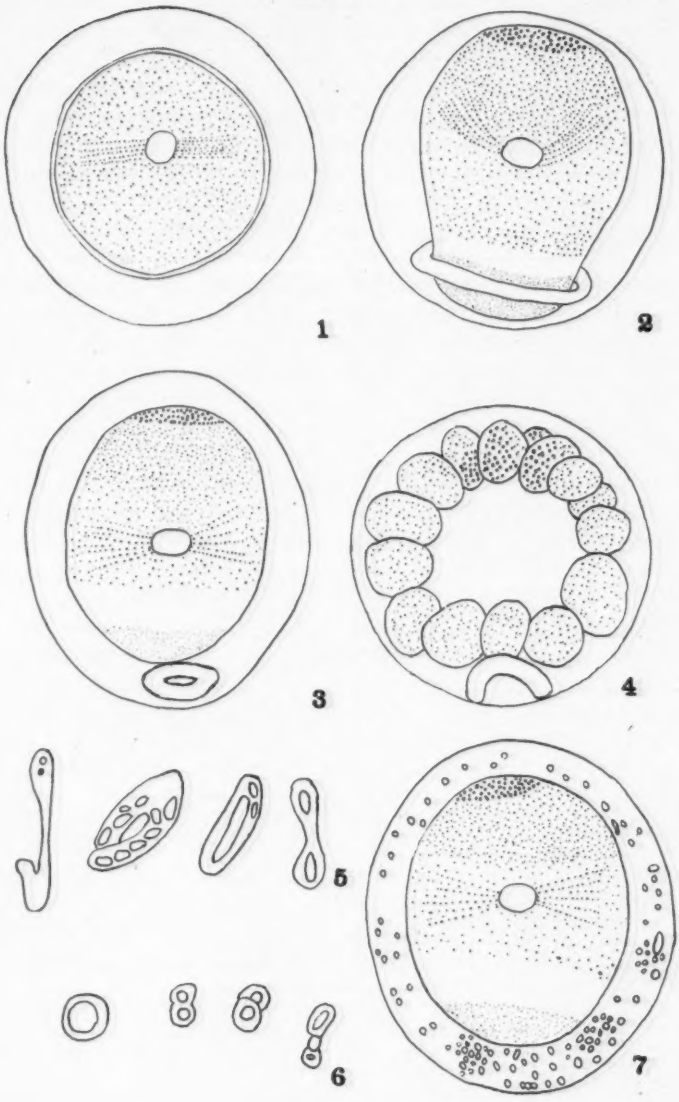
PLATE I

Parechinus microtuberculatus

1. Normal egg 30 minutes after insemination, showing ectoplasmic layer.
2. Egg centrifuged 30 minutes after insemination (for 3 minutes at 5,000 $\times$ g), showing ectoplasmic ring encircling heavy pole of elongate egg.
3. Same egg 10 minutes later; ectoplasmic ring lying in perivitelline space.
4. Same egg, early blastula; ectoplasmic ring still present.
5. Various forms of ectoplasmic ring when centrifuged off before first cleavage.
6. Various forms of ectoplasmic ring centrifuged off during 2-cell stage.
7. Egg kept and centrifuged in calcium-free medium, then put into sea water. Ectoplasmic material has precipitated in perivitelline space as small refringent bodies. Fertilized at 10:37; placed in calcium-free medium at 10:42; centrifuged from 10:47–10:51; then put in sea water; drawn at 10:55.

PLATE I



are put in *calcium-free* sea water five minutes after fertilization (*i.e.*, time to allow the fertilization membrane to harden), in order to disintegrate the ectoplasmic layer, and are then centrifuged in a mixture of this solution and isotonic sugar (same density as the eggs), the ring does not form. If the eggs after removal from the centrifuge are left in the same solution (*i.e.*, minus Ca) or transferred to calcium-free sea water, the perivitelline space remains perfectly clear and there is no ectoplasmic layer on the surface of the eggs. If the eggs, on removal from the centrifuge, are put in normal sea water, the perivitelline space becomes filled with many small refringent spherical or oval bodies (Fig. 7), the precipitation product of the ectoplasmic material in the presence of calcium. On return to calcium-free sea water, these are again dissolved and can be precipitated again in the presence of calcium. The ectoplasmic material, after being centrifuged off as a ring, may also be dissolved in calcium-free sea water and be precipitated again as scattered spherules when returned to sea water. Similar refringent spheres arising from the ectoplasmic layer have been described in eggs treated with acid sea water by Gray (1924, 1931) and by Moore (1928, 1932). The ectoplasmic layer, according to these two authors, reacts chemically like a calcium proteinate. Whatever its exact chemical nature, my experiments show that the ectoplasmic layer is a very definite external layer or membrane, highly unstable in the absence of calcium, easily peeled off by centrifugal force, and readily reformed under certain conditions after removal.

### Protoplasm

The effects of centrifugal force on the protoplasm of the fertilized eggs of *Parechinus, Paracentrotus, Sphærechinus,* and *Arbacia pustu-*

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Photographs 1–8

1. *Parechinus microtuberculatus.* Fertilized at 11:42; centrifuged from 11:52–11:58; taken at 11:59. Note ectoplasmic ring encircling heavy (small) pole of egg.

2. *Arbacia punctulata.* Centrifuged 8 minutes after fertilization. Ectoplasmic crescent within broken fertilization membrane.

3. *Parechinus microtuberculatus.* Fertilized at 10:15; centrifuged from 10:18–10:22 (5,000 × g). Eggs fragmented in many small pieces.

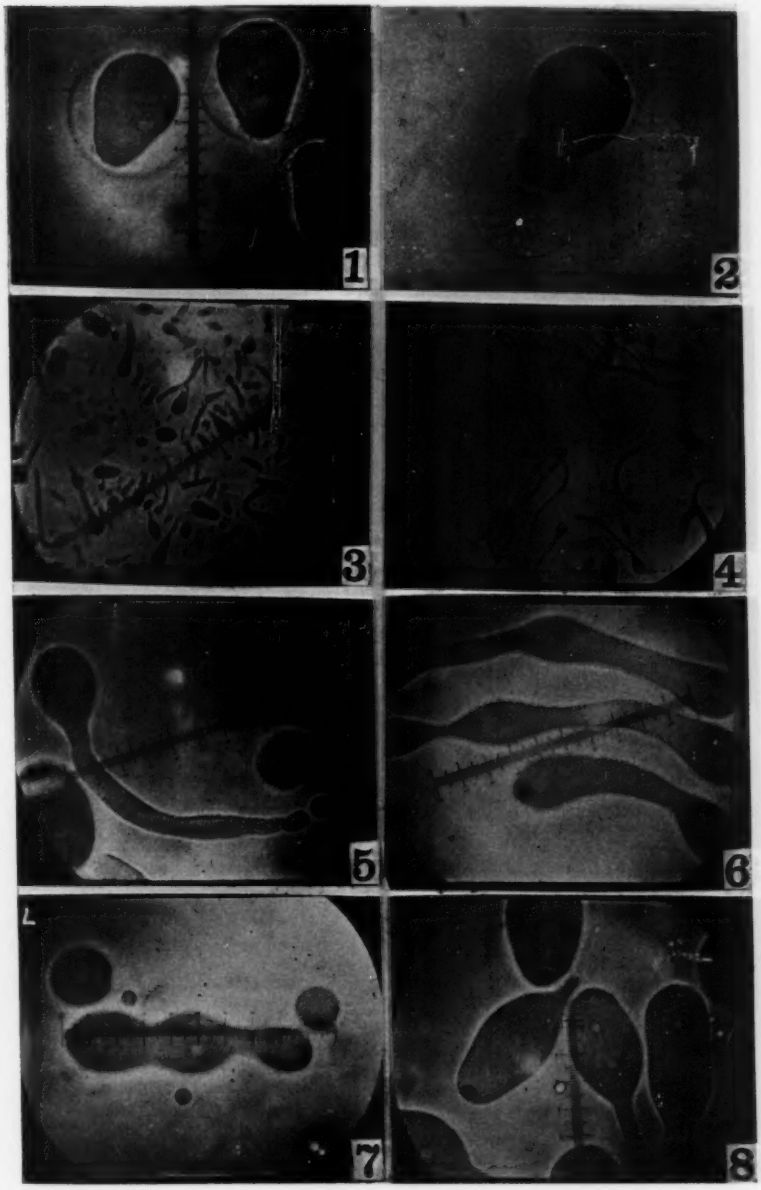
4. *Sphærechinus granularis.* Centrifuged 21 minutes after fertilization for 3 minutes (5,000 × g). Streamer stage, great elongation.

5. *Sphærechinus granularis.* Much elongated egg breaking up into small fragments.

6. *Parechinus microtuberculatus.* Fertilized at 8:50; centrifuged from 9:01–9:05. Taken at 9:15. Note bulge in egg where male aster is.

7. *Parechinus microtuberculatus.* Centrifuged 9 minutes after fertilization. Female nucleus below oil, ♂ nucleus in center; note bulge here.

8. *Parechinus microtuberculatus.* Fertilized at 10:15; centrifuged from 10:23–10:27. Egg more contracted; ♂ and ♀ nuclei present.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*losa* have been found to be practically the same as described for *Arbacia punctulata* (E. B. Harvey, 1933*b*). There is a period soon after fertilization (about 1½ to 6 minutes at 18° C.) when the eggs break very readily into many small pieces (Photograph 3); even slight shaking in the case of *Parechinus* and *Paracentrotus* at this period is sufficient to thoroughly fragment the eggs. The fertilization membrane at this time is elastic and easily broken; it sometimes stretches to twice its original diameter before breaking. After about six minutes it becomes quite tough and inelastic, and must be removed from the eggs (at 2 minutes after insemination by shaking) for experiments with the later stages. During or before the monaster stage (6–30 minutes after insemination, the time varying with the different species), the eggs elongate greatly, forming long streamers sometimes fourteen times the original diameter of the egg (Photographs 4–6); the streamers of the *Parechinus* and *Paracentrotus* egg are longer than those of the *Sphærechinus* egg. The streamers retract very quickly immediately upon removal from the centrifuge; a *Parechinus* egg which was stretched to 1300 μ retracted to 455 μ in five minutes. After the first few minutes they retract more gradually, often becoming spherical or nearly so, within an hour; they frequently break into small fragments, especially in the intermediate zone between the light and heavy poles which tend to remain somewhat intact (Photographs 4, 5). During the streak stage, the eggs elongate less and just before cleavage very little. The fertilized eggs of *Parechinus* and *Paracentrotus* break at all stages *more* readily than unfertilized eggs of the same batch centrifuged at the same time; the fertilized eggs of *Sphærechinus* break at all stages *less* readily than control unfertilized eggs. *Arbacia punctulata* is intermediate in this respect (E. B. Harvey, 1933*b*). In all the forms, the fertilized

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PLATE II

Parechinus microtuberculatus

8. Normal egg showing size of pronuclei at time of union; 14 minutes after insemination.

9. Egg fertilized at 9:45; centrifuged (5,000 × g) from 9:55–10:00; drawn at 10:10. Male nucleus measures 8 μ; ♀ nucleus 13 μ.

10. Same egg drawn at 10:25. Male and ♀ nuclei measure 13 μ.

11. Same egg drawn at 10:35. Male nucleus measures 19 μ; ♀ nucleus 8 μ. Note nucleolus in ♂ nucleus.

12. Same egg drawn at 10:40. Nuclei have fused and measure 20 μ.

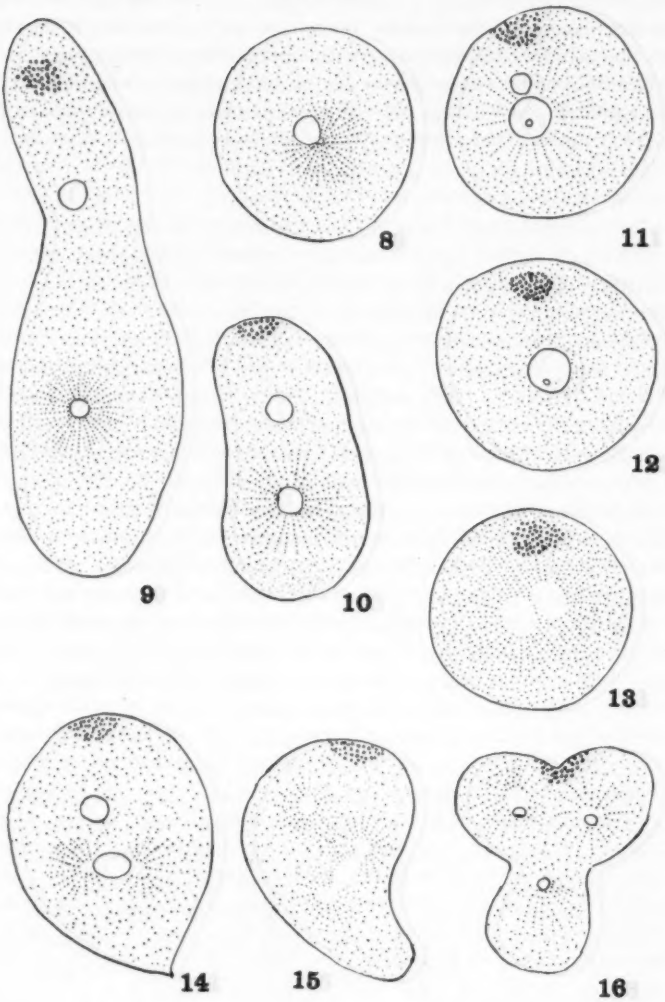
13. Same egg drawn at 11:45. Cleavage amphiaster.

14. Another egg. Fertilized at 11:28; centrifuged from 11:37–11:41; drawn at 12:07. Male nucleus forms amphiaster before union with ♀.

15. Another egg. Fertilized at 10:05; centrifuged from 10:12–10:16; drawn at 11:15. Male nucleus forms amphiaster, ♀ monaster.

16. Same egg at 11:22; tripolar cleavage.

PLATE II



eggs stratify less readily than the control unfertilized eggs, showing that they are more viscous; this difference is slight soon after fertilization but becomes more marked in later stages. As has been pointed out in the case of *Arbacia punctulata*, the greater ease of breaking must be due to surface differences between the fertilized and unfertilized eggs. The differences cannot, however, be due to the ectoplasmic layer, since this is scarcely formed at the time of maximal fragmentation and in later stages is thrown off by the centrifugal force as previously described.

## Nuclei

In the eggs greatly elongated by centrifugal force (during or just before the monaster stage), the two pronuclei are thrown apart, the female pronucleus going to the light pole and the male to the heavy pole. The presence of the male nucleus in the heavy end of the eggs (of *Parechinus*, *Sphærechinus*, and *Paracentrotus*) is often made apparent first by the sperm aster. A pronounced bulging of the egg in this region (Photographs 6, 7) lends support to the generally accepted view that asters are of considerable rigidity and form regions of greater gelation. That the two nuclei should go to different poles is not surprising in view of the fact that at this time the male nucleus consists practically entirely of chromatin material in a very condensed form, whereas the female nucleus consists of the same material together with a large amount of other more fluid material. The spermatozoa are themselves heavier than the entire egg since they are thrown by centrifugal force to the bottom of a tube in the same medium in which the eggs remain suspended.

In normal uncentrifuged sea urchin eggs, the two pronuclei unite soon (6–15 minutes) after insemination while still very unequal in size (Fig. 8). By centrifuging the eggs, the two nuclei become so widely separated that it takes considerable time (an hour or more) for them to come together, which they do both because of the contraction of the egg protoplasm and by movement on their part through the protoplasm. During this time, the male nucleus increases enormously in size, so that it becomes not only of equal size with the female but often much larger; it frequently acquires a nucleolus while enlarging (Figs. 9–12, Photographs 7, 8). There is considerable variation in the actual size attained by the two nuclei; the female nucleus of *Parechinus* is about 13 $\mu$ in diameter, increasing to 16 $\mu$ before dissolving; the male nucleus has been observed to increase to 19 $\mu$. The discrepancy in size is often much greater owing to the fact that the female nucleus may get smaller (before disappearing) at the same time that the nearby male nucleus is getting larger (Fig. 11). The fusion nucleus of the *Parechinus* egg usually measures 16 $\mu$ at its maximum, but has been observed to increase to 22 $\mu$.

The male nucleus does not increase greatly in size (usually under 10 $\mu$) when it is alone in a fragment obtained by centrifuging either before or after fertilization. This is probably due to the greater packing of granules in the surrounding protoplasm in these fragments as compared with the protoplasm surrounding the male nucleus when present (together with the female pronucleus) near the centripetal pole. The large size (22 $\mu$) sometimes attained by the fusion nucleus in the clear quarter eggs of *Arbacia punctulata* where the surrounding medium is granule-free (E. B. Harvey, 1932) lends support to this explanation.

The importance of the time factor in controlling the size relations of the two nuclei is shown in the case of the eggs of many animals, annelids, mollusks, and even the closely related *Asterias* (Wilson and Mathews, 1895). The sperm here enters or may enter the egg before the polar bodies are given off and during the delay thus caused, the sperm nucleus increases in size until it is equal to the female. The two nuclei also become of equal size in the sea urchin *Toxopneustes,* if their union is delayed by treatment with ether (Wilson, 1902).

After the female and large male pronuclei unite as above described (Fig. 12), an amphiaster is formed and the egg cleaves normally (Fig. 13). Frequently the male centrosome divides before union of the two pronuclei, so that the advancing male nucleus is accompanied by an amphiaster instead of a monaster (Fig. 14). This is the case normally in *Asterias* and many annelids and mollusks and often occurs in etherized *Toxopneustes* eggs (Wilson, 1902). This amphiaster becomes or gives rise to the cleavage amphiaster after union of the two pronuclei, and normal cleavage follows. In some cases, the female nucleus decreases in size and disappears before union with the male, and the ensuing mitotic figure is then a triaster, the female nucleus being replaced by a monaster (Figs. 15, 16). The changes taking place in the male and female pronuclei and in the accompanying asters seem to be quite independent of each other and the variations occurring normally in different species can be duplicated in one species by varying the time relations of the events; this is accomplished in these elongate eggs produced by centrifugal force.

With a greater centrifugal force, the elongate egg can be broken into two parts, the lighter fragment containing the female nucleus and the denser fragment the male nucleus (Fig. 17). In the majority of experiments with both *Parechinus* and *Paracentrotus,* continuous observation of many of these half-eggs gave the following results. The fragment containing the female nucleus does not divide (Figs. 18–21). The nucleus enlarges (to about 16 $\mu$), disappears, a large monaster

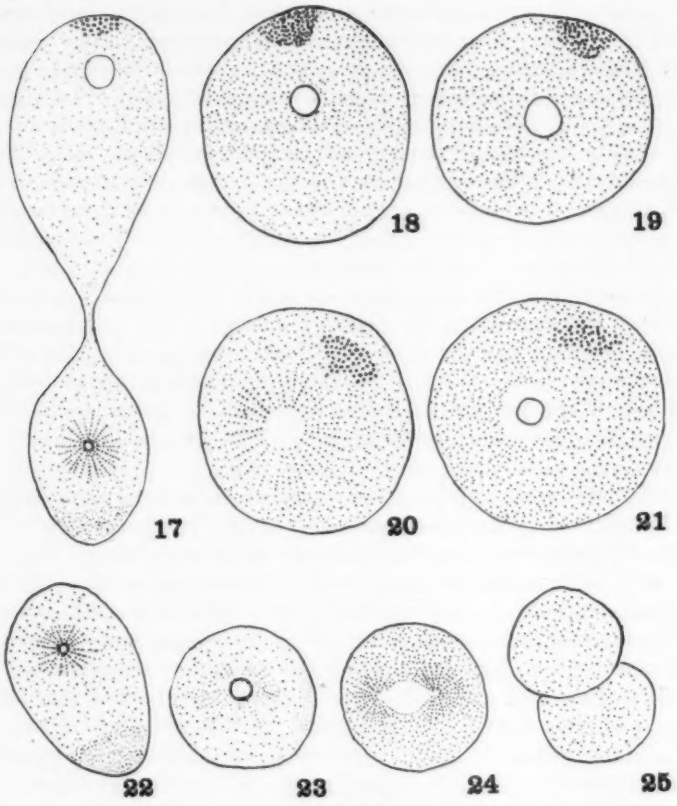



PLATE III

Parechinus microtuberculatus

17. Segregation of ♂ and ♀ nuclei into separate fragments; division center associated with ♂ nucleus. Fertilized at 10:05; centrifuged from 10:12–10:16; drawn at 10:26.

18–21. Development of fragment with ♀ nucleus.

18. Egg fertilized at 9:12; centrifuged from 9:15–9:19; fragment drawn at 9:35. Nucleus measures 13 μ.

19. Same fragment at 10:10. Nucleus measures 16 μ.

20. Same fragment at 10:45. Monaster.

21. Same fragment at 10:50. Nucleus back. Monaster again like Fig. 20 at 11:10; amœboid at 12:00.

22–25. Development of fragment with ♂ nucleus.

22. Fertilized at 10:05; centrifuged from 10:12–10:16; fragment drawn at 10:30. Sperm aster.

23. Same fragment at 10:40. Larger ♂ nucleus.

24. Same fragment at 11:15. Sperm amphiaster.

25. Same fragment at 11:30. Cleavage.

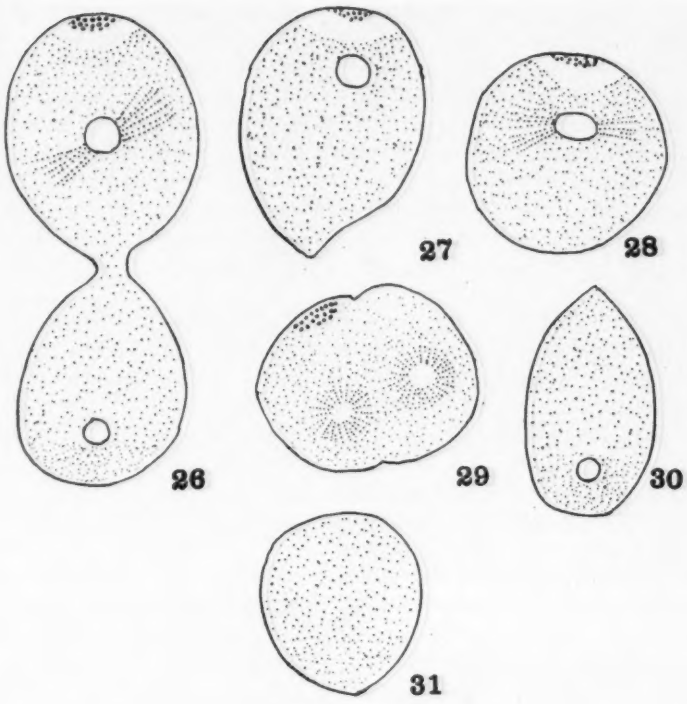



PLATE IV

Paracentrotus lividus

26. Segregation of ♂ and ♀ nuclei into separate fragments; division center associated with ♀ nucleus. Fertilized at 10:20; centrifuged from 10:37–10:41; drawn at 11:00.

27–29. Development of fragment with ♀ nucleus.

27. Egg fertilized at 12:08; centrifuged from 12:25–12:30; fragment drawn at 12:40. Nucleus enlarges to 16 μ.

28. Same fragment at 12:55. Rays from ♀ nucleus.

29. Same fragment at 1:20. Division.

30–31. History of corresponding fragment from same egg with ♂ nucleus.

30. Drawn at 12:40. No rays from ♂ nucleus.

31. Drawn at 12:55. Nucleus gone.

appears, the nucleus may later be reformed and again break down to form a monaster, often with a granular interior. This process may be repeated many times and finally the cell often becomes amœboid, but does not cleave. The fragment containing the male nucleus, on the

other hand, develops quite normally; the nucleus enlarges (to about 10 $\mu$), then fades out and an amphiaster appears and the cell cleaves normally (Figs. 22–25). The active division center appears to be associated with the male nucleus; and the female nucleus, lacking it, is unable to form an amphiaster. This seems curious in view of the fact that the female nucleus can function alone and form an amphiaster in parthenogenesis. It was also found that in *Arbacia punctulata,* where the egg was separated inside the fertilization membrane, the white half containing the female nucleus alone frequently developed together with the red half containing the male nucleus alone, though this was not always the case (E. B. Harvey, 1933*b*). It is interesting to find that the eggs separated by hand with a cotton thread, so that one part contained the female nucleus and the other the male (in Ziegler's (1898) experiments with the same egg, *Parechinus*), behaved in exactly the same way as these separated by centrifugal force.

In two lots of *Paracentrotus* eggs, both from the same batch, centrifuged 17 minutes after insemination, the reverse of the usual occurrence took place in many eggs, though not in all. In the whole egg partially separated, the astral rays were associated with the female nucleus and none with the male (Fig. 26). When these eggs were completely separated, the amphiaster arose in connection with the female nucleus and this fragment divided (Figs. 27–29); no rays formed around the male nucleus, but it grew smaller and disappeared and the cell never divided (Figs. 30, 31). This same lot of eggs centrifuged earlier (15 minutes after insemination) gave the usual result, the amphiaster associated with the male nucleus; when centrifuged later (19 minutes) the two nuclei were not separated. It may be that there is a critical period of short duration when the active division center, arising

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Photographs 9–15

9. *Sphærechinus granularis.* Centrifuged in spindle stage one hour after fertilization. Note oil drops in amphiaster in clear layer.

10. *Sphærechinus granularis.* Shows division plane through oil perpendicular to stratification.

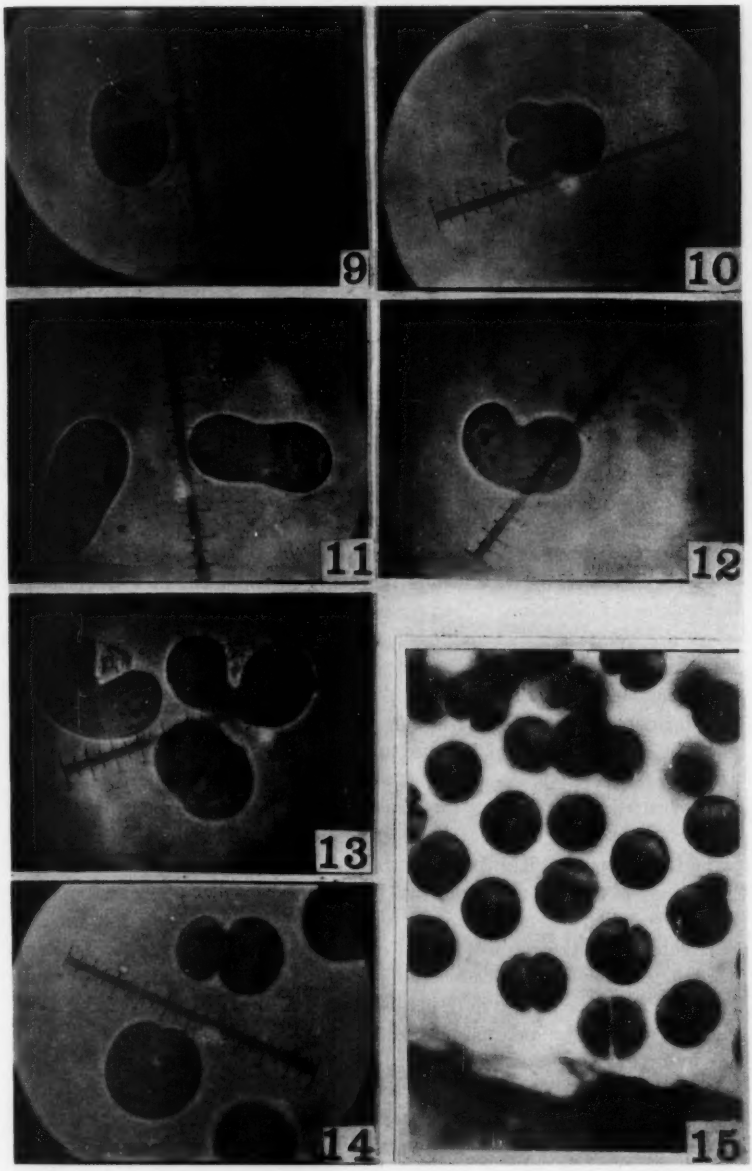
11. *Parechinus microtuberculatus.* Centrifuged just before cleavage. Note oil in astral rays.

12. Similar egg, showing division plane starting parallel with stratification and in no relation to mitotic figure, which is outlined by oil drops.

13. Similar eggs a little later. Note also disorganized ectoplasmic material in cleavage furrow.

14. *Paracentrotus lividus.* Centrifuged just before cleavage one hour after fertilization. Cleavage plane (in upper cell) has separated off clear cell which contains mitotic figure.

15. *Arbacia punctulata.* Centrifuged just before cleavage on centrifuge-microscope. Note central cell cleaved into one clear and one pigmented blastomere; the spindle is in the clear area.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as it usually does, in connection with the male nucleus, becomes independent of it or more closely associated with the female nucleus.

### Amphiaster and Cleavage

When the eggs of any of the sea urchins are centrifuged after the amphiaster has formed, many of the oil drops are stopped in their passage to the light pole by the rays of the asters. In many cases the asters are completely outlined by the oil drops. This is particularly striking in eggs like *Sphaerechinus* and *Arbacia* where the mitotic figure lies in a perfectly clear area; this clear band is optically empty except for two small areas where the oil drops are enmeshed in the asters (Photograph 9). It is also quite striking even when the mitotic figure lies among granules as in the *Parechinus* egg (Photographs 11, 12). This demonstrates very clearly that the mitotic figure is a very definite structure consisting of more rigid or gelated material than the surrounding medium. The presence of the spindle and asters in the clear zone in fixed and stained material has been demonstrated by Spooner (1911) in the *Arbacia* egg.

If centrifuged for a sufficiently long time at a sufficiently high speed, the mitotic figure takes up its position under the oil cap with its long axis parallel with the stratification and the cleavage plane is perpendicular to the stratification (Photograph 10). This may occur even while eggs are rotating at fairly high speeds, as can be observed with the centrifuge-microscope (E. B. Harvey, 1933*b*). If, however, the eggs are centrifuged rapidly after the elongation preliminary to cleavage, the eggs orient with the long axis along the axis of centrifugal force (unless held in another position by the surfaces of the centrifuge-microscope slide). The spindle is still thrown into the light zone under the oil cap and oriented as before, but the cleavage plane may come in ***parallel with*** the stratification in the short axis and often in the exact position that it would have come in had the mitotic figure not been moved (Photographs 11–15). The cleavage plane thus bears no relation to the new position of the spindle, but to its former position. The change in the surface correlated with the initial location of the spindle is apparently sufficient for the formation of the cleavage plane in its original position. Whether chromosomes have been left behind and function in the formation of the cleavage plane has not been determined, as they cannot be seen in the living egg. The cleavage furrow comes in in this way, that is, without relation to the new position of the spindle, both in eggs while rotating, as can be seen with the centrifuge-microscope, and after the centrifugal force has been removed. The breaking strain due to centrifugal force also comes in this region, for at this time the eggs tend to break at or

near the equator into two fairly equal parts, whereas in earlier stages they break into unequal parts. A further investigation of the relation of the mitotic figure to the cleavage plane in the transparent eggs of *Parechinus* and *Paracentrotus* will be undertaken shortly, using the centrifuge-microscope.

## Later Development of Eggs and Fragments

It has been noted above that when eggs are centrifuged with the fertilization membranes intact, they develop normally although the ectoplasmic layer is thrown off; this is apparently reformed if the eggs are protected by the fertilization membrane. They do not develop in any large percentage, however, if centrifuged during cleavage; this is no doubt owing to the disturbances of the mitotic figure (and chromosomes) mentioned above. It has also been noted previously that fragments without nuclei do not develop, and fragments containing only the female nucleus do not usually develop. Whole eggs and fragments other than those mentioned may develop and form swimming blastulae and plutei. In many cases, however, loose clusters of cells are formed owing to the lack of an ectoplasmic layer. This is true for eggs centrifuged soon after fertilization when the fertilization membranes are not properly formed or are destroyed, and for eggs from which membranes have been removed, and which have been centrifuged at any stage after fertilization. This applies to both whole eggs and fragments. No study has been made of the later development of individual eggs or egg fragments, but observation of experimental lots shows that failure to develop depends chiefly on the falling apart of the cells due to the lack of an ectoplasmic layer and that both large (from whole eggs) and small (from fragments) plutei occur in lots centrifuged at any stage after insemination.

## Summary

1. The ectoplasmic layer is *added on* to the surface of the egg of *Arbacia pustulosa* on fertilization.

2. The ectoplasmic layer can be thrown off the fertilized eggs of *Parechinus microtuberculatus, Paracentrotus lividus, Sphærechinus granularis, Arbacia pustulosa,* and *A. punctulata* by centrifugal force as a ring or crescent which lies in the perivitelline space.

3. The ring is not formed in absence of calcium, but the dissolved ectoplasmic material is precipitated when the eggs are returned to sea water as refringent spherules in the perivitelline space.

4. The ectoplasmic layer is reformed on eggs with fertilization membranes and the eggs develop normally.

5. Soon after insemination (1½–6 minutes), all the species studied break into many very small pieces; during the monaster stage (6–30 minutes), they form long streamers; later, elongate dumb-bells. The fertilized eggs of *Parechinus* and *Paracentrotus* break more readily at all stages than unfertilized eggs, those of *Sphærechinus* less readily; the fertilized eggs of all species stratify less rapidly than the unfertilized.

6. The female pronucleus is driven by centrifugal force to the light pole and the male pronucleus to the heavy pole of the elongate eggs.

7. The male pronucleus may become much larger than the female before fusion; the size of the male nucleus depends on the time before union and the density of the surrounding protoplasm.

8. An egg may be broken into two fragments, one containing the female and the other the male nucleus. Usually the former forms a monaster and does not develop, the latter an amphiaster followed by normal cleavage. In two lots of *Paracentrotus* eggs, the reverse took place; the division center was associated with the female nucleus and this fragment divided while the other fragment with the male nucleus did not divide.

9. The spindle is thrown to the light pole and cleavage usually comes in through its equator, perpendicular to the stratification. If centrifuged just before cleavage, the cleavage plane may come in parallel with the stratification and in no relation to the new position of the spindle.

10. Many whole eggs centrifuged after fertilization develop normally and also many fragments thus obtained. Failure to develop is due to the lack of an ectoplasmic layer which causes the cells to fall apart, or to the absence of one or both nuclei.

## LITERATURE CITED

CHAMBERS, R., 1921. Microdissection Studies. III. Some problems in the maturation and fertilization of the echinoderm egg. *Biol. Bull.*, **41**: 318.

GLASER, O., 1914. The Change in Volume of Arbacia and Asterias Eggs at Fertilization. *Biol. Bull.*, **26**: 84.

GLASER, O. C., 1924. Fertilization, Cortex and Volume. *Biol. Bull.*, **47**: 274.

GRAY, J., 1924. The Mechanism of Cell Division. I. The forces which control the form and cleavage of the eggs of Echinus esculentus. *Proc. Camb. Phil. Soc.*, **1**: 164.

GRAY, J., 1931. Experimental Cytology. Cambridge University Press.

HARVEY, E. B., 1932. The Development of Half and Quarter Eggs of Arbacia punctulata and of Strongly Centrifuged Whole Eggs. *Biol. Bull.*, **62**: 155.

HARVEY, E. B., 1933*a*. Development of the Parts of Sea Urchin Eggs Separated by Centrifugal Force. *Biol. Bull.*, **64**: 125.

HARVEY, E. B., 1933*b*. Effects of Centrifugal Force on Fertilized Eggs of Arbacia punctulata as Observed with the Centrifuge-microscope. *Biol. Bull.*, **65**: 389.

HARVEY, E. N., 1933. Flattening of Marine Eggs under the Influence of Gravity. *Jour. Cell and Comp. Physiol.*, **4**: 35.

HERBST, C., 1900. Über das Auseinandergehen von Furchungs- und Gewebezellen in kalkfreiem Medium. *Arch. f. Entw.-Mech.*, **9**: 424.

JUST, E. E., 1928. Studies of Cell Division. II. The period of maximum susceptibility to dilute sea water in the cleavage cycle of the fertilized egg of Arbacia. *Physiol. Zoöl.*, **1**: 26.

MOORE, A. R., 1928. On the Hyaline Membrane and Hyaline Droplets of the Fertilized Egg of the Sea Urchin, Strongylocentrotus purpuratus. *Protoplasma*, **3**: 524.

MOORE, A. R., 1930. Fertilization and Development without Membrane Formation in the Egg of the Sea Urchin Strongylocentrotus purpuratus. *Protoplasma*, **9**: 9.

MOORE, A. R., 1932. The Dependence of Cytoplasmic Structures in the Egg of the Sea Urchin on the Ionic Balance of the Environment. *Jour. Cell. and Comp. Physiol.*, **2**: 41.

SPOONER, G. B., 1911. Embryological Studies with the Centrifuge. *Jour. Exper. Zoöl.*, **10**: 23.

WILSON, E. B., 1902. Experimental Studies in Cytology. II. Some phenomena of fertilization and cell-division in etherized eggs. *Arch. f. Entw-Mech.*, **13**: 353.

WILSON, E. B., AND A. P. MATHEWS, 1895. Maturation, Fertilization and Polarity in the Echinoderm Egg. New light on the "quadrille of the centers." *Jour. Morph.*, **10**: 319.

ZIEGLER, H. E., 1898. Experimentelle Studien über die Zelltheilung. I. Die Zerschnürung des Seeigeleier. II. Furchung ohne Chromosomen. *Arch. f. Entw.-Mech.*, **6**: 249.

ZIEGLER, H. E., 1903. Experimentelle Studien über die Zelltheilung. IV. Die Zelltheilung der Furchungszellen von Beroe und Echinus. *Arch. f. Entw.-Mech.*, **16**: 155.